GENETIC ENGINEERING SALT TOLERANCE IN CROP PLANTS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. application nos. 60/078,474 and 60/116,111, which are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

Environmental stress due to salinity is one of the most serious factors limiting the productivity of agricultural crops, which are predominantly sensitive to the presence of high concentrations of salts in the soil. Large terrestrial areas of the world are affected by levels of salt inimical to plant growth. It is estimated that 35-45% of the 279 million hectares of land under irrigation is presently affected by salinity. This is exclusive of the regions classified as arid and desert lands, (which comprises 25% of the total land of our planet). Salinity has been an important factor in human history and in the life spans of agricultural systems. Salt impinging on agricultural soils has created instability and has frequently destroyed ancient and recent agrarian societies. The Sumerian culture faded as a power in the ancient world due to salt accumulation in the valleys of the Euphrates and Tigris rivers. Large areas of the Indian subcontinent have been rendered unproductive through salt accumulation and poor irrigation practices. In this century, other areas, including vast regions of Australia, Europe, southwest USA, the Canadian prairies and others have seen considerable declines in crop productivity.

Although there is engineering technology available to combat this problem, though drainage and supply of high quality water, these measures are extremely costly. In most of the cases, due to the increased need for extensive agriculture, neither improved irrigation efficiency nor the installation of drainage systems is applicable. Moreover, in the arid and semi-arid regions of the world water evaporation exceeds precipitation. These soils are inherently high in salt and require vast amounts of irrigation to become productive. Since irrigation water contains dissolved salts and minerals, an application of water is also an application of salt that compounds the salinity problem.

Increasing emphasis is being given to modify plants to fit the restrictive growing conditions imposed by salinity. If economically important crops could be manipulated and made salt resistant, this land could be farmed resulting in greater sales of seed and greater yield of useful crops. Conventional breeding for salt tolerance has been attempted for a long time. These breeding practices have been based mainly on the following strategies:

a) the use of wide crosses between crop plants and their more salt-tolerant wild relatives [1], b) screening and selecting for variation within a particular phenotype [2], c) designing new phenotypes through recurrent selection [3]. The lack of success in generating tolerant varieties (given the low number of varieties released and their limited salt tolerance) [4] would suggest that conventional breeding practices are not enough and that in order to succeed a breeding program should include the engineering of transgenic crops [5].

Several biochemical pathways associated with stress tolerance have been characterized in different plants and a few of the genes involved in these processes have been identified and in some cases the possible role of proteins has been investigated in transgenic/overexpression experiments. Several compatible solutes have been proposed to play a role in osmoregulation under stress. Such compatible solutes, including carbohydrates [6], amino acids [7] and quaternary N-compounds [8] have been shown to increase osmoregulation under stress. Also, proteins that are normally expressed during seed maturation (LEAs, Late Embriogenesis Abundant proteins) have been suggested to play a role in water retention and in the protection of other proteins during stress. The overexpression of LEA in rice provided a moderate benefit to the plants during water stress [9,10]. A single gene (sod2) coding for a Na^+/H^+ antiport has been shown to confer sodium tolerance in fission yeast [11,12], although the role of this plasma membrane-bound protein appears to be only limited to yeast. One of the main disadvantages of using this gene for transformation of plants is associated with the typical problems encountered in heterologous gene expression, i.e. incorrect folding of the gene product, targeting of the protein to the target membrane and regulation of the protein function.

Plants that tolerate and grow in saline environments have high intracellular salt levels. A major component of the osmotic adjustment in these cells is accomplished by ion uptake. The utilization of inorganic ions for osmotic adjustment suggests that salt-tolerant plants must be able to tolerate high levels of salts within their cells. However, enzymes extracted from these plants show high sensitivity to salt. The sensitivity of the cytosolic enzymes to salt would suggest that the maintenance of low cytosolic sodium concentration, either by compartmentation in cell organelles or by exclusion through the plasma membrane, must be necessary if the enzymes in the cell are to be protected from the inimical effects of salt.

Plant cells are structurally well suited to the compartmentation of ions. Large membrane-bound vacuoles are the site for a considerable amount of sequestration of ions

and other osmotically active substances. A comparison of ion distribution in cells and tissues of various plant species indicates that a primary characteristic of salt tolerant plants is their ability to exclude sodium out of the cell and to take up sodium and to sequester it in the cell vacuoles. Transport mechanisms could actively move ions into the vacuole, removing the potentially harmful ions from the cytosol. These ions, in turn, could act as an osmoticum within the vacuole, which would then be responsible for maintaining water flow into the cell. Thus, at the cellular level both specific transport systems for sodium accumulation in the vacuole and sodium extrusion out of the cell are correlated with salt tolerance.

SUMMARY OF THE INVENTION

We have isolated the first such system of intracellular salt management. We identified the presence of a functional vacuolar Na^+/H^+ antiport in the vacuolar membrane of higher plants [13,14,15,16,17,18].

We have demonstrated the Na^+/H^+ antiport function in isolated tonoplast membranes and in intact vesicles and we showed that the activity of antiport molecules was salt dependent. Neither a protein sequence nor a gene encoding the antiport were identified in previously published work. We have now identified nucleic acid molecules coding for plant Na^+/H^+ antiports, the nucleic acid molecules and polypeptides produced by the nucleic acid molecules being the subject of the present invention. These polypeptides are useful for the extrusion of sodium ions from the cytosol, either through the accumulation of sodium ions into the vacuoles or into the extracellular space, thus providing the most important trait for salt tolerance in plants. These nucleic acid molecules, preferably genes, are useful for the engineering of salt tolerant plants by transformation of salt-sensitive crops overexpressing one or more of these nucleic acid molecules under the control of constitutively active promoters or under the control of conditionally-induced promoters. Agrobacterium tumefaciens-mediated transformation or particle-bombardment-mediated transformation are useful for depending upon the plant species.

The invention includes an isolated nucleic acid molecule encoding a PNHX transporter polypeptide, or a fragment of a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

The invention also relates to an isolated nucleic acid molecule encoding a THX transporter polypeptide, PNHX transporter polypeptide, or a fragment of a polypeptide

having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell, comprising a nucleic acid molecule selected from the group consisting of:

(a) a nucleic acid molecule that hybridizes to all or part of a nucleic molecule in [SEQ ID NO:1], [SEQ ID NO:3], [SEQ ID NO:17], [SEQ ID NO:19], or a complement thereof under moderate or high stringency hybridization conditions, wherein the nucleic acid molecule encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell;

(b) a nucleic acid molecule degenerate with respect to (a), wherein the nucleic molecule encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

The hybridization conditions preferably comprise moderate (also called intermediate) or high stringency conditions selected from the conditions in Table 4.

The invention also includes an isolated nucleic acid molecule encoding a THX transporter polypeptide or a PNHX transporter polypeptide, or a fragment of a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerances in a cell, comprising a nucleic acid molecule selected from the group consisting of:

(a) the nucleic acid molecule of the coding strand shown in [SEQ ID NO:1], [SEQ ID NO:3], [SEQ ID NO:17], [SEQ ID NO:19] or a complement thereof;

(b) a nucleic acid molecule encoding the same amino acid sequence as a nucleotide sequence of (a); and

(c) a nucleic acid molecule having at least 17% identity with the nucleotide sequence of (a) and which encodes a THX transporter polypeptide or the PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity.

The THX transporter polypeptide or the PNHX transporter polypeptide preferably comprises an AtNHX transporter polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell. The nucleic acid molecule may comprise all or part of a nucleotide sequence in [SEQ ID NO:1], [SEQ ID NO:3], [SEQ ID NO:17] or [SEQ ID NO:19] (or the coding region therof).

The invention also includes an AtNHX nucleic acid molecule isolated from *Arabidopsis thaliana*, or a fragment thereof encoding a transporter polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

Another aspect of the invention relates to a recombinant nucleic acid molecule comprising a nucleic acid molecule and a constitutive promoter sequence or an inducible promoter sequence, operatively linked so that the promoter enhances transcription of the nucleic acid molecule in a host cell.

The nucleic acid molecule preferably comprises genomic DNA, cDNA or RNA. In another aspect, the nucleic acid molecule is chemically synthesized. The nucleic acid molecule is preferably isolated from *Arabidopsis thaliana.*

The nucleic acid molecule preferably encodes a TNHX transporter polypeptide or PNHX transporter polypeptide that is capable of extruding monovalent cations out of the cytosol of a cell to provide the cell with increased salt tolerance, wherein the monovalent cations are selected from at least one of the group consisting of sodium, lithium and potassium. The cell preferably comprises a plant cell. The monovalent cations are preferably extruded into a vacuole or into the extracellular space.

The invention also includes an isolated nucleic acid molecule comprising a nucleic acid molecule selected from the group consisting of 8 to 10 nucleotides of the nucleic acid molecules described above, 11 to 25 nucleotides of the nucleic acid molecules described above, and 26 to 50 nucleotides of the nucleic acid molecules described above.

The invention also includes an isolated oligonucleotide comprising at least about 10 nucleotides from a sequence selected from the group consisting of 5'-GCCATGTTGGATTCTCTAGTGTCG-3 [SEQ ID NO:11], 5'-CCGAATTCTCAAAGCTTTTCTTCCACG-3' [SEQ ID NO:12], 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3' [SEQ ID NO:13], 5'-GCCATGTTGGATTCTCTAGTGTCG-3 [SEQ ID NO:14], 5'-CCGAATTCTCAAAGCTTTTCTTCCACG-3' [SEQ ID NO:15], 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3' [SEQ ID NO:16] or another oligonucleotide described in this application.

Another aspect of the invention relates to a vector comprising a nucleic acid molecule of the invention. The vector preferably comprises a promoter selected from the

group consisting of a super promoter, a 35S promoter of cauliflower mosaic virus, a drought-inducible promoter, an ABA-inducible promoter, a heat shock-inducible promoter, a salt-inducible promoter, a copper-inducible promoter, a steroid-inducible promoter and a tissue-specific promoter.

The invention also includes a host cell comprising a recombinant nucleic acid molecule of the invention, or progeny of the host cell.

The host cell is preferably selected from the group consisting of a fungal cell, a yeast cell, a bacterial cell, a microorganism cell and a plant cell. The plant, a plant part, a seed, a plant cell or progeny thereof preferably comprises the recombinant nucleic acid molecule of the invention. The plant part preferably comprises all or part of a leaf, a flower, a stem, a root or a tuber. The plant, plant part, seed or plant cell is preferably of a species selected from the group consisting of potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, sorgum, alfalfa, salicornia and the plant species or types in Table 5.

The plant, plant part, seed or plant cell preferably comprises a dicot plant or a monocot plant.

The invention also relates to a method for producing a recombinant host cell capable of expressing the nucleic acid molecule of the invention, the method comprising introducing into the host cell a vector of the invention. The invention also includes a method of producing a genetically transformed plant which expresses TNHX or PNHX transporter polypeptide, comprising regenerating a genetically transformed plant from a plant cell, seed or plant part of the invention. In one method, the genome of the host cell also includes a functional TNHX or PNHX gene. In another method, the genome of the host cell does not include a functional TNHX or PNHX gene. The invention also includes a transgenic plant produced according to a method of the invention.

Another aspect of the invention relates to a method for expressing a TNHX or PNHX transporter polypeptide in the host cell of the invention, a the plant, plant part, seed or plant cell of the invention, the method comprising culturing the host cell under conditions suitable for gene expression. A method for producing a transgenic plant that expresses elevated levels of PNHX transporter polypeptide relative to a non-transgenic plant, comprising transforming a plant with the vector of the invention. The invention also relates to an isolated polypeptide encoded by and/or produced from a nucleic acid molecule of the

invention, or the vector of the invention.

The invention also relates to an isolated PNHX transporter polypeptide or a fragment thereof having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell. The polypeptide of the invention preferably comprises an AtNHX transporter polypeptide. The polypeptide of the invention preferably comprises all or part of an amino acid sequence in [SEQ ID NOS: 2, 4, 6, 8, 18, or 20] (figure 1). The invention also includes a polypeptide fragment of the AtNHX transporter polypeptide of the invention, or a peptide mimetic of the AtNHX transporter polypeptide, having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell. The polypeptide fragment of the invention, preferably consists of at least 20 amino acids, which fragment has Na^+/H^+ transporter activity and is capable of increasing salt tolerance in a cell. The fragment or peptide mimetic of the invention is preferably capable of being bound by an antibody to a polypeptide of the invention. In one embodiment, the polypeptide of the invention is recombinantly produced.

The invention also includes an isolated and purified transporter polypeptide comprising the amino acid sequence of a TNHX transporter polypeptide or a PNHX transporter polypeptide, wherein the transporter polypeptide is encoded by a nucleic acid molecule that hybridizes under moderate or stringent conditions to a nucleic acid molecule in [SEQ ID NOS: 1, 3, 5, 7, 17, or 19] (figure 1), a degenerate form thereof or a complement. The invention also includes a polypeptide comprising a sequence having greater than 28% sequence identity to a polypeptide of the invention (preferably a polypeptide in figure 1, such as [SEQ ID NOS: 2, 4, 6, 8, 18, or 20]).

The polypeptide of the invention, preferably comprises a Na^+/H^+ transporter polypeptide. The polypeptide is preferably isolated from *Arabidopsis thaliana*.

The invention also includes an isolated nucleic acid molecule encoding polypeptide of the invention (preferably a polypeptide in figure 1: [SEQ ID NOS: 2, 4, 6, 8, 18, or 20]).

Another aspect of the invention relates to an antibody directed against a polypeptide of the invention. The antibody of the invention, preferably comprises a monoclonal antibody or a polyclonal antibody.

The invention also relates to an isolated nucleic acid molecule encoding a TNHX transporter polypeptide or a PNHX transporter polypeptide, or a fragment of a polypeptide

having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell, comprising a nucleic acid molecule selected from the group consisting of:

(a) a nucleic acid molecule that hybridizes to all or part of a nucleic molecule in [SEQ ID NO:5], [SEQ ID NO:7], [SEQ ID NO:9] or to a nucleic acid molecule comprising about nucleotides 1-1487 of [SEQ ID NO:9], or a complement thereof under moderate or high stringency hybridization conditions, wherein the nucleic acid molecule encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell;

(b) a nucleic acid molecule degenerate with respect to (a), wherein the nucleic molecule encodes a TNHX polypeptide, a PNHX polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

(c) the nucleic acid molecule of the coding strand shown in [SEQ ID NO:5], [SEQ ID NO:7], [SEQ ID NO:9], nucleotides 1-1487 of [SEQ ID NO:9], or an isolated nucleic acid molecule including about 1614 nucleic acids including [SEQ ID NO:5], [SEQ ID NO:7], nucleotides 1 to 1487 of the nucleic acid molecule in [SEQ ID NO:9] or the complement thereof;

(d) a nucleic acid molecule encoding the same amino acid sequence as a nucleotide sequence of (c); and

(e) a nucleic acid molecule having at least 17% sequence identity with the nucleotide sequence of (c) and which encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell.

The invention also includes a polypeptide produced from a nucleic acid molecule of the invention. The invention includes a polypeptide comprising (a) the amino acid sequence in [SEQ ID NO:6], [SEQ ID NO:8], [SEQ ID NO:10]; (b) amino acids 1 to 496 of [SEQ ID NO:10]; and (c) a sequence having greater than 28% homology to the polypeptide in (a) or (b). The invention includes a polypeptide comprising a Na^+/H^+ transporter polypeptide capable of increasing salt tolerance in a cell. The invention also includes a DNA molecule encoding the polypeptides of the invention.

The invention relates to a method of producing a genetically transformed plant which expresses or overexpresses a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide having Na^+/H^+ transporter activity and capable of increasing salt tolerance in a cell and wherein the plant has increased salt tolerance, comprising:

a) cloning or synthesizing a TNHX nucleic acid molecule, a PNHX nucleic acid molecule or a nucleic acid molecule which codes for a Na^+/H^+ transporter polypeptide, wherein the polypeptide is capable of providing salt tolerance to a plant;

b) inserting the nucleic acid molecule in a vector so that the nucleic acid molecule is operably linked to a promoter;

c) inserting the vector into a plant cell or plant seed;

d) regenerating the plant from the plant cell or plant seed, wherein salt tolerance in the plant is increased compared to a wild type plant.

The invention includes a transgenic plant produced according to a method of the invention.

The nucleic acid molecules have several uses which will be discussed in more detail below. The nucleic acid molecules and the polypeptides are used in a method for protecting a plant from the adverse affects of a saline environment by incorporating a nucleic acid molecule for salt tolerance and/or the polypeptide of the invention into a plant. The nucleic acid molecules of the invention are also useful for the identification of homologous nucleic acid molecules from plant species, preferably salt tolerant species and genetically engineering salt tolerant plants of agricultural and commercial interest.

The invention relates to isolated nucleic acid molecules encoding a polypeptide for extrusion of sodium ions from the cytosol of a cell to provide the cell with salt tolerance. The nucleic acid molecules preferably comprise the nucleotide sequence in figure 1(a) or (b). The nucleic acid molecules may be DNA or RNA. The nucleic acid molecules may be used to transform a cell selected from the group consisting of a plant cell, a yeast cell and a bacterial cell. The sodium ions are extruded into a vacuole or out of the cell. The nucleic acid molecules encode a Na^+/H^+ exchanger polypeptide.

In a preferred embodiment, the nucleic acid molecules are isolated from *Arabidopsis thaliana*.

The invention includes an isolated nucleic acid molecule, comprising the DNA sequence in figure 1(a), (b), (c)(i), (c)(ii), (d) or (e). The invention also relates to an isolated nucleic acid molecule, comprising a sequence having greater than 17% homology to the sequences of the invention described in the preceding paragraphs.

In an alternate embodiment, the nucleic acid molecule consists of a sequence selected from the group consisting of 8 to 10 nucleotides of the nucleic acid molecules of the invention, 11 to 25 nucleotides of the nucleic acid molecule and 26 to 50 nucleotides of the nucleic acid molecules. These nucleic acid molecules hybridize to nucleic acid molecules described in the preceding paragraphs.

The nucleic acid molecule of the invention may have a sense or an antisense sequence.

In another embodiment, the invention is an isolated oligonucleotide consisting of a sequence selected from the group consisting of 5'-GCCATGTTGGATTCTCTAGTGTCG-3 [SEQ ID NO:11], 5'- CCGAATTCTCAAAGCTTTTCTTCCACG-3' [SEQ ID NO:12], 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3' [SEQ ID NO:13], an oligonucleotide with an antisense sequence of 5'-GCCATGTTGGATTCTCTAGTGTCG-3 [SEQ ID NO:14], an oligonucleotide with an antisense sequence of 5'-CCGAATTCTCAAAGCTTTTCTTCCACG-3' [SEQ ID NO:15] and an oligonucleotide with an antisense sequence of 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3' [SEQ ID NO:16]. The invention includes an isolated oligonucleotide consisting of 5 to 15 nucleotides of these oligonucleotides. The invention includes an isolated oligonucleotide consisting of a sequence homologous to the oligonucleotide of claim 15 or claim 16.

In an alternate embodiment, the invention is an expression vector comprising a nucleic acid molecule of the invention. The expression vector preferably consists of a promoter selected from the group consisting of a super promoter, a 35S promoter of cauliflower mosaic virus, a drought-inducible promoter, an ABA-inducible promoter, a heat shock-inducible promoter, a salt-inducible promoter, a copper-inducible promoter, a steroid-inducible promoter and a tissue-specific promoter.

The invention is a polypeptide produced from the nucleic acid molecules of the invention. The invention is also a polypeptide produced from the expression vector. The

polypeptide is used for extrusion of sodium ions from the cytosol of a cell to provide the cell with salt tolerance.

In a preferred embodiment, the polypeptide has the amino acid sequence in figure 1(a)-(e). The polypeptides may be homologous to the polypeptide in figure 1(a)-(e). In an alternate embodiment, the polypeptides comprise a sequence having greater than 28% homology to the polypeptide in figure 1(a)-(e). The polypeptides are Na^+/H^+ exchanger polypeptides.

The polypeptides are preferably isolated from *Arabidopsis thaliana.*

The invention includes peptides consisting of at least 5 amino acids of the polypeptides described in the preceding paragraphs. In another embodiment, the peptides consist of 41 to 75 amino acids of the polypeptides described in the preceding paragraphs.

The invention also includes isolated nucleic acid molecules encoding the polypeptides of the invention. The isolated nucleic acid molecule preferably encodes the polypeptide of figure 1(a)-(e).

The polypeptides of the invention that extrude sodium ions from the cytosol of a cell to provide the cell with salt tolerance, preferably consist of an amiloride binding domain. The amiloride binding domain is between amino acids 82 to 90 in both AtNHX1 and AtNHX2. in figure 1(a)-(e) and between amino acids 58 to 66 in both AtNHX3 and AtNHX4 in figures (d) and (e).

The invention also includes a monoclonal antibody or polyclonal antibody directed against a polypeptide of the invention.

Another embodiment of the invention includes a transformed microorganism comprising an isolated nucleic acid molecule of the invention. The invention also includes a transformed microorganism including an expression vector.

The invention includes a plant cell transformed with a nucleic acid molecule of the invention. The invention also includes a yeast cell transformed with the nucleic acid molecule of the invention. In another embodiment, the invention is a plant, plant part or seed, generated from a plant cell transformed with a nucleic acid molecule of the invention. The invention also relates to a plant, plant part, seed or plant cell transfected with a nucleic acid molecule of the invention. The plant, plant part, seed or plant cell is preferably selected from a species selected from the group consisting of potato, tomato, brassica,

cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, sorgum, alfalfa and salicornia and other plants in Table 5.

The invention also includes a method for producing a polypeptide of the invention by culturing a plant, plant part, seed or plant cell of the invention and recovering the expressed polypeptide from the culture.

The invention includes an isolated nucleic acid molecule encoding a polypeptide capable of extruding monovalent cations from the cytosol of a cell to provide the cell with increased salt tolerance. The nucleic acid molecule preferably includes the nucleotide sequence in figure 1(a)-(e). The nucleic acid molecule is preferably DNA or RNA. The cell is preferably a plant cell, a yeast cell or a bacterial cell. The monovalent cations are preferably sodium, lithium or potassium. The monovalent cations are preferably extruded into a vacuole or out of the cell. The nucleic acid molecules preferably encode a Na^+/H^+ exchanger polypeptide. The nucleic acid molecule is preferably isolated from *Arabidopsis thaliana*.

The invention also includes an isolated nucleic acid molecule, including a sequence having greater than 17% homology to a sequence referred to in the preceding paragraph.

The invention also includes a nucleic acid molecule of 8 to 10 nucleotides, 11 to 25 or 26 to 50 nucleotides of a nucleic acid molecule of the invention.

The invention also includes a nucleic acid molecule which nucleic acid molecule hybridizes a nucleic acid molecule of the invention. The nucleic acid molecule comprises a sense or an antisense sequence.

The invention also includes an isolated oligonucleotide including a sequence selected from the group consisting of 5'-GCCATGTTGGATTCTCTAGTGTCG-3 [SEQ ID NO:11], 5'- CCGAATTCTCAAAGCTTTTCTTCCACG-3' [SEQ ID NO:12], 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3' [SEQ ID NO:13], 5'-GCCATGTTGGATTCTCTAGTGTCG-3 [SEQ ID NO:14], 5'-CCGAATTCTCAAAGCTTTTCTTCCACG-3' [SEQ ID NO:15] and 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3' [SEQ ID NO:16] or 5 to 15 nucleotides of one of these oligonucleotides. The invention also includes an isolated oligonucleotide having a sequence homologous to one of these oligonucleotides.

The invention also includes an expression vector including a nucleic acid molecule

of the invention. The expression vector preferably comprises a promoter selected from the group consisting of a super promoter, a 35S promoter of cauliflower mosaic virus, a drought-inducible promoter, an ABA-inducible promoter, a heat shock-inducible promoter, a salt-inducible promoter, a copper-inducible promoter, a steroid-inducible promoter and a tissue-specific promoter.

The invention also includes a polypeptide produced from a nucleic acid molecule or expression vector of the invention. The invention also includes a polypeptide for extrusion of monovalent cations ions from the cytosol of a cell to provide the cell with salt tolerance. The invention also includes a polypeptide including the amino acid sequence in figure 1(a)-(e) or a polypeptide homologous to one of these sequences. The invention also includes a polypeptide including a sequence having greater than 28% homology to one of these polypeptides. The polypeptide is preferably a Na^+/H^+ exchanger polypeptide isolated from *Arabidopsis thaliana*. The invention also includes a peptide including at least 5 amino acids or 41 to 75 amino acids of the polypeptide of the invention. The invention also includes nucleic acid molecules these polypeptides.

The invention includes a polypeptide for extrusion of monovalent cations ions from the cytosol of a cell to provide the cell with salt tolerance, including, but not necessarily having, an amiloride binding domain.

Another aspect of the invention relates to a monoclonal or polyclonal antibody directed against a polypeptide of the invention.

Another variation includes a transformed microorganism including an isolated nucleic acid molecule of the invention. The transformed microorganism preferably includes an expression vector of the invention.

A plant cell, yeast cell transformed or transfected with a nucleic acid molecule or a plant, plant part or seed, generated from the plant cell. The plant, plant part, seed or plant cell is preferably from a species selected from the group consisting of potato, tomato, brassica, cotton, sunflower; strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, sorgum, alfalfa, salicornia and other plants in Table 5. The invention also includes a method for producing a peptide, by culturing the plant, plant part, seed or plant cell and recovering the expressed peptide from the culture.

The invention includes a nucleic acid molecule that encodes all or part of a polypeptide capable of extruding monovalent cations from the cytosol of a cell to provide

the cell with salt tolerance, wherein the sequence hybridizes to the nucleic acid molecule of all or part of [SEQ ID NO:1] or [SEQ ID NO:3], [SEQ ID NO:17], [SEQ ID NO:19] , figure 5(b) or a nucleic acid molecule including nucleotides 1-1487 of figure 5(b) under low, medium and high stringency conditions. The high stringency conditions preferably comprise a wash stringency of selected from the group of hybridization and wash stringencies in Table 4.

The invention includes an isolated nucleic acid molecule encoding a polypeptide capable of extruding monovalent cations from the cytosol of a cell to provide the cell with salt tolerance, including the nucleic acid molecule in figure 5(b). The invention also includes an isolated nucleic acid molecule encoding a polypeptide capable of extruding monovalent cations from the cytosol of a cell to provide the cell with salt tolerance, including nucleotides 1 to 1487 of the nucleic acid molecule in figure 5(b).

Another aspect of the invention relates to an isolated nucleic acid molecule including about 1640 (or preferably about 1600 or 1700) nucleic acids encoding a polypeptide capable of extruding monovalent cations from the cytosol of a cell to provide the cell with salt tolerance, the nucleic acid molecule including nucleotides 1 to 1487 (or preferably about nucleotides 1 to 1470, 1480, 1490 or 1500) of the nucleic acid molecule in figure 5(b). The molecule is preferably DNA or RNA. The cell is preferably selected from the group consisting of a plant cell, a yeast cell and a bacterial cell. The molecule preferably encodes a Na^+/H^+ exchanger polypeptide. The nucleic acid molecule is preferably isolated from *Arabidopsis thaliana*.

The invention also includes the nucleic acid molecule in figure 5(b) or a nucleic acid molecule having greater than 17% homology to the sequence in 5(b). The invention includes polypeptides produced from this one of these nucleic acid molecules. The invention also relates to a polypeptide including the amino acid sequence in figure 5(b) or amino acids 1 to 496 of figure 5(b). (note: polypeptide including 1 to 496 is preferably about 530, 540 or 550 amino acids, most preferably about 538 amino acids) or a homologous polypeptide, preferably having greater than 28% homology. The polypeptide is preferably a Na^+/H^+ exchanger polypeptide, isolated from *Arabidopsis thaliana*. The invention also includes a DNA molecule encoding one of these polypeptides.

The invention includes an isolated nucleic acid molecule encoding a polypeptide capable of extruding monovalent cations from the cytosol of a cell to provide the cell with

salt tolerance, including at least one of the nucleic acid molecules in figure 1(c). The molecule is preferably DNA or RNA. The cell is preferably selected from the group consisting of a plant cell, a yeast cell and a bacterial cell and encodes a Na^+/H^+ exchanger polypeptide isolated from *Arabidopsis thaliana*.

The invention includes an isolated nucleic acid molecule, including the nucleic acid molecule in figure 1(c)(i) or 1(c)(ii) or a polypeptide produced from a nucleic acid molecule of the invention. The invention also includes a polypeptide including the amino acid sequence in figure 1(c)(i) or 1(c)(ii) or homologous to this polypeptide, preferably having greater than 28% homology. The polypeptide is preferably a Na^+/H^+ exchanger polypeptide, isolated from *Arabidopsis thaliana*. The invention includes a DNA molecule encoding one of these polypeptides.

It will be clear to one skilled in the art that the sequences in figures 1(c) and 5 are useful in isolating other salt tolerant nucleic acid molecules (for example probes may be made from the sequences in figures 1(c) and 5), preparing transgenic plants and performing many of the other methods of the invention that are described with respect to sequences in figures 1(a), (b), (d) and (e). Variants and modifications of figure 1(c) and figure 5 sequences are also included within the invention as are methods using varied or modified sequences (the same preferred percentages of identity and sequence described with respect to figures 1(a), (b), (d) and (e) also apply to figures 1(c) and 5). Nucleic acid molecules including a portion of the nucleic acid molecule in figure 5 preferably include about nucleotides 1-1487 (or a partial sequence thereof, preferably starting from the coding region, which will be apparent to a skilled person, at about nucleotide 286). The nucleotide sequence including all or part of sequence in figure 1(c) or figure (5) will be preferably about 1614 nucleotides in length (or the 1614 nucleotides minus part or all of the 5' untranslated region nucleotides). The nucleic acid molecules are most preferably 1600 to 1620 nucleotides in length. Polypeptides including a portion of the nucleic acid molecule in figure 5 preferably include about amino acids 1 to 496 (or a partial sequence thereof) in figure 5. The sequences encoding all or part of the polypeptide in figure 5 or encoding a polypeptide corresponding to either of the nucleic acid molecule sequences in figure 1(c) are preferably about 538 amino acids in length and preferably about 60 kda in length. Preferred polypeptides are about 530-550 amino acids in length.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention are described in relation to the drawings in which:

Figure 1. (a) Shows the nucleic acid molecule that is [SEQ ID NO:1] and the polypeptide that is [SEQ ID NO:2].

In a preferred embodiment, the figure shows isolated *AtNHX1* cDNA encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana* showing cDNA sequence and the corresponding amino acid sequence for AtNHX1. Twelve transmembrane domains are present, a conserved amiloride-binding domain is present, and a relatively hydrophilic C-terminal region is also present. The predicted open reading frame begins at nucleotide 286. The amino acids are centred below the corresponding codon and are numbered on the left;

(b) Shows the nucleic acid molecule that is [SEQ ID NO:3] and the polypeptide that is [SEQ ID NO:4].

In a preferred embodiment, the figure shows isolated AtNHX2 cDNA encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana* showing cDNA sequence and the corresponding predicted amino acid sequence for AtNHX2. The predicted open reading frame begins at nucleotide 61. The amino acids are centred below the corresponding codon and are numbered on the left;

(c) (i) Shows the nucleic acid molecule that is [SEQ ID NO:5] and the polypeptide that is [SEQ ID NO:6]. (ii) Shows the nucleic acid molecule that is [SEQ ID NO:7] and the polypeptide that is [SEQ ID NO:8].

In a preferred embodiment, the figure shows AtNHX3 partial cDNA sequences. The amino acids are centred below the corresponding codon and are numbered on the left (i) 5' sequence of the partial AtNHX3 cDNA and amino acid sequence; (ii) In a preferred embodiment, the figure shows 3' sequence of the partial AtNHX3 cDNA and amino sequence;

(d) Shows the nucleic acid molecule that is [SEQ ID NO:17] and the polypeptide that is [SEQ ID NO:18].

In a preferred embodiment, the figure shows isolated AtNHX3 cDNA encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana* showing cDNA sequence and the

corresponding predicted amino acid sequence for AtNHX3. The predicted open reading frame begins at nucleotide 67. The amino acids are centred below the corresponding codon and are numbered on the left. (e) Isolated AtNHX4 cDNA encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana* showing cDNA sequence [SEQ ID NO:19] and the corresponding predicted amino acid sequence [SEQ ID NO:20] for AtNHX4. The predicted open reading frame begins at nucleotide 55. The amino acids are centred below the corresponding codon and are numbered on the left.

(e) Shows the nucleic acid molecule that is [SEQ ID NO:19] and the polypeptide that is [SEQ ID NO:20].

In a preferred embodiment, the figure shows isolated AtNHX4 cDNA encoding a Na^+/H^+ exchanger from *Arabidopsis thaliana*.

Figure 2. **(a)** Alignment of the predicted amino acid sequences of Arabidopsis AtNHX1, from *Arabidopsis thaliana* with other Na^+/H^+ exchangers from other organisms. Sequences were aligned using the Clustal W program [19] using default parameters (fixed gap penalty=10, floating gap penalty=10, protein weight matrix BLOSUM62). Sequences and GenBank accession numbers are: ScNHX1, late endosomal Na^+/H^+ exchanger S. cerevisiae (GenBank accession #927695); CeNHE1, C. elegans (GenBank accession # 3877723; HsNHE6, Homo sapiens mitochondrial Na^+/H^+ exchanger (GenBank accession # 2944237); (b) Alignment of the predicted amino acid sequences of Arabidopsis AtNHX1, AtNHX2 and AtNXH3 cDNAs from *Arabidopsis thaliana.* Sequences were aligned using the Clustal W program [19] using default parameters (fixed gap penalty=10, floating gap penalty=10, protein weight matrix BLOSUM62). (c) Alignment of the predicted amino acid sequences of Arabidopsis AtNHX3 and AtNXH4 cDNAs from *Arabidopsis thaliana.* Sequences were aligned using the Clustal W program [19] using default parameters (fixed gap penalty=10, floating gap penalty=10, protein weight matrix BLOSUM62); **(b)** Alignment of the predicted amino acid sequences of AtNHX1, AtNHX2 and AtNHX3 cDNAs from *Arabidopsis thaliana*. Sequences were aligned using the Clustal W prgoram using default parameters (fixed gap penalty=10, floating gap penalty=10, protein weight matrix BLOSUM62); **(c)** Alignment of the predicted amino acid sequences of AtNHX3 and AtNHX4 cDNAs from *Arabidopsis thaliana*. Sequences were aligned using the Clustal W program using default parameters (fixed gap penalty=10, floating gap penalty=10, protein weight matrix BLOSUM62).

Figure 3. A Southern blot of *Arabidopsis* genomic DNA. Genomic DNA (10 μg per lane)

was digested with various restriction enzymes, separated on a 1.0% agarose gel, transferred onto a GeneScreen Plus membrane (Amersham), and hybridized to a radiolabelled AtNHX1 cDNA as described in Materials and Methods. Restriction enzymes used were; **C**, ClaI; **E**, ECoRI; **X**, XbaI; **H**, HindIII.

Figure 4. RNA blot of AtNHX1 expression in different tissues. Total RNA (40 μg) was separated on a 1.0% agarose gel, transferred to a GeneScreen Plus membrane (Amersham) and hybridized to a radiolabelled AtNHX1 cDNA probe as described in Materials and Methods. Tissues in each lane were as follows: **1**, mature leaf; **2**, flower (including sepals); **3**, infloresence stem; **4**, seedling shoot; **5**, seedling root.

Figure 5. (a) and (b) show the nucleic acid molecule that is [SEQ ID NO:9] and the polypeptide that is [SEQ ID NO:10].

In a preferred embodiment, (a) and (b) show modified arabidopsis sodium/proton antiporter cDNA and polypeptide sequence.

Figure 6. RNA blot comparing transcript levels in *Arabidopsis thaliana* leaf tissue from wild type and different transgenic lines overexpressing *AtNHX1.* RNA was extracted from 4 week-old plants. Total RNA (30 μg per lane) was separated on a 1.0 % agarose gel, transferred to a GeneScreen Plus membrane (Amersham) and hybridized to a radiolabelled AtNHX1 cDNA probe as described in Materials and Methods. An endogenous 2.1 kb transcript was detected in the transgenic lines as well as in wild type. An overexpressed1.8 kb transcript was only seen in the transgenic lines. The 1.8 kb transcript corresponds to the open reading frame coding for AtNHX1, lacking the 5'- and 3'-untranslated regions present in the original cDNA (2.1 kb). Ribosomal RNA (rRNA) was used to confirm equal loading of the gels, as seen by methylene-blue staining of the blot.

wt: wild-type; **X1-2', X1-3' and X1-4':** independent transgenic lines.

Figure 7. Twenty 3-week old kanamycin-resistant *Arabidopsis thaliana* plants for each of the 3 independent transgenic lines (X1.2' , X1.3' and X1.4') transformed with *AtNHX1,* as well as 20 wild-type plants of the same age were used for assessment of salt tolerance. Plants were watered with 25 ml of 1/8 strength MS salts (control solution) supplemented with different concentrations of NaCl. The following schedule was used for a total of 16 days, at which point pictures of representative plants were taken: **a)** wild-type: A=0mM NaCl, B=50mM NaCl, C=100mM NaCl, D=150mM NaCl, E=200mM NaCl; **b)** X1.2'

transgenic line: A=0mM NaCl, B=50mM NaCl, C=100mM NaCl, D=150mM NaCl, E=200mM NaCl; **c)** X1.3' transgenic line; **d)** X1.4' transgenic line: A=0mM NaCl, B=50mM NaCl, C=100mM NaCl, D=150mM NaCl, E=200mM NaCl; **e)** wild type: A=0mM NaCl, E=200mM NaCl vs. transgenic strain 2': A=0mM NaCl, E=200mM NaCl; **f)** wild type: A=0mM NaCl, E= 200mM NaCl vs. transgenic strain 4': A=0mM NaCl, E=200mM NaCl; **g)** wild type: A=0mM NaCl, E=200mM NaCl vs. transgenic strain 2': A=0mM NaCl, E=200mM NaCl and transgenic strain 4': A=0mM NaCl, E=200mM NaCl.

Treatments:

A) watered with a control solution (1/8 MS strength solution, 0mM NaCl) eight times (once every two days)

B) watered with a control solution supplemented with 50mM NaCl eight times (once every two days)

C) watered twice (once every two days) with a control solution supplemented with 50mM NaCl, then with a control solution supplemented with 100mM NaCl six times (once every two days).

D) watered twice (once every two days) with a control solution supplemented with 50mM NaCl, then with a control solution supplemented with 100mM NaCl twice (once every two days) followed by a control solution supplemented with 150mM NaCl four times (once every two days).

E) watered twice (once every two days) with a control solution supplemented with 50mM NaCl, then with a control solution supplemented with 100mM NaCl twice (once every two days) followed by a control solution supplemented with 150mM NaCl twice (once every two days) and a control solution supplemented with 200 mM NaCl twice (once every two days).

Figure 8. (a) shows [SEQ ID NO:21] (b) shows [SEQ ID NO:22] (c) shows [SEQ ID NO:23] (d) shows [SEQ ID NO:24] (e) shows [SEQ ID NO:25] (f) shows [SEQ ID NO:26] (g) shows [SEQ ID NO:27] (h) shows [SEQ ID NO:28].

In preferred embodiments, (a)-(h) show sequences from Table 2: **(a)** GenBank Accession No. 3850064 569 a.a.; **(b)** GenBank Accession No. 927695 633 a.a.; **(c)** GenBank Accession No. C91832 378 bp mRNA EST; **(d)** GenBank Accession No. C91861 268 bp mRNA EST; **(e)** GenBank Accession No. AU032544 380 bp mRNA EST; **(f)**

GenBank Accession No. AA660573 596 bp mRNA EST; **(g)** GenBank Accession No. L44032 522 bp mRNA STS; **(h)** GenBank Accession No. T75860 (EST) 330 bp mRNA EST.

DETAILED DESCRIPTION OF THE INVENTION

Salt Tolerance Nucleic Acid Molecules and Polypeptides

The invention relates to nucleic acid molecules and polypeptides which increase salt tolerance in cells and plants. PNHX polypeptides are plant Na^+/H^+ transporter polypeptides that are capable of increasing and enhancing salt tolerance in a cell, preferably a plant cell. These transporters (also referred to as exchangers, antiports or antiporters) extrude monovalent cations (preferably potassium ions or lithium ions, most preferably sodium ions) out of the cytosol. The cations are preferably extruded into the vacuoles or extracellular space. The affinity for particular ions varies between transporters. The listed preferences refer to the cations that are most likely to be abundant in the cytosol and therefore most likely to be extruded. It is not necessarily a reflection of transporter affinity for particular cations. The *PNHX* nucleic acid molecules which encode PNHX polypeptides are particularly useful in producing transgenic plants which have increased salt tolerance compared to a wild type plant.

It will also be apparent that there are polypeptide and nucleic acid molecules from other organisms, such as yeast, microorganisms, fish, birds or mammals, that are similar to PNHX polypeptides and nucleic acid molecules. The entire group of Na^+/H^+ transporter polypeptides and nucleic acid molecules that are capable of increasing salt tolerance in a cell (including PNHX and AtNHX polypeptides and nucleic acid molecules) are collectively referred to as ("TNHX polypeptides" and "TNHX nucleic acid molecules"). TNHX polypeptides are Na^+/H^+ transporters that are capable of increasing salt tolerance in a cell, preferably a plant cell, because they extrude monovalent cations (preferably potassium ions or lithium ions, most preferably sodium ions) out of the cytosol.

The role of TNHX and PNHX nucleic acid molecules and polypeptides in maintaining salt tolerance was not shown before this invention. The ability of these compounds to increase salt tolerance of transgenic host cells (particularly plant cells) and transgenic plants compared to wild type cells and plants was unknown.

PNHX and TNHX polypeptides need not necessarily have the primary function of providing salt tolerance. All nucleotides and polypeptides which are suitable for use in the

methods of the invention, such as the preparation of transgenic host cells or transgenic plants, are included within the scope of the invention. Genomic clones or cDNA clones are preferred for preparation of transgenic cells and plants.

In a preferred embodiment, the invention relates to cDNAs encoding Na^+/H^+ exchangers from *Arabidopsis thaliana*. The cDNA sequences and the corresponding amino acid sequences for AtNHX1, AtNHX2, AtNHX3 and AtNHX4 are presented in Figure 1. AtNHX1 and AtNHX2 are homologs that are physically located at different places in the genome. The invention also includes splice variants of the nucleic acid molecules as well as polypeptides produced from the molecules. For example, AtNHX3 and AtNHX4 are homologs of AtNHX1 and AtNHX2. AtNHX3 and AtNHX4 are identical for a long sequence beginning at the N-terminus. This indicates that the difference in sequence at the C-terminus is due to alternative splicing of a nucleic acid molecule (also known as splicing variants). This allows a single nucleic acid molecule to produce varying polypeptides.

Characterization of Salt Tolerance Nucleic Acid Molecules and Polypeptides

The longest open reading frame of 1614 base pairs in AtNHX1 encodes a polypeptide of 538 amino acids with a predicted molecular weight ("MW") of about 60 Kda. A comparison of this full length cDNA with the *Arabidopsis* genome sequence (A-TM021B04.4) revealed the presence of 13 introns and 14 exons. This polypeptide encoded by the open reading frame was about 19% larger than the sequence predicted by the Arabidopsis genomic sequence (A_TM021B04.4). This sequence encodes the full length exchanger given that the cDNA region immediately upstream of the start codon contains predicted stop codons in all three reading frames. In addition, a transcript of approximately 2 kb, which corresponds roughly in size to the predicted mRNA for AtNHX1, was observed on RNA blots. Based on the amino acid sequence of AtNHX1, 12 transmembrane domains are predicted, a conserved amiloride-binding domain is present, and a relatively hydrophilic C-terminal region is also predicted. AtNHX1 shows some similarity at the amino acid level to Na^+/H^+ exchangers isolated from a variety of organisms ranging from yeast (about 27% identity) to humans (about 20%). A second salt tolerance cDNA and polypeptide, AtNHX2, was obtained from *Arabidopsis thaliana* (Figure 1(b)). We characterized a third salt tolerance nucleic acid molecule, AtNHX3, by obtaining 5' and 3' cDNA and N-terminal and C-terminal sequences from *Arabidopsis thaliana* (Figure 1(c)). In one variation, the invention includes DNA sequences (and the corresponding polypeptide) including at least one of the sequences shown in figure 1(c) in a nucleic acid molecule of preferably about:

less than 1000 base pairs, less than 1250 base pairs, less than 1500 base pairs, less than 1750 base pairs, less than 2000 base pairs, less than 2250 base pairs, less than 2500 base pairs, less than 2750 base pairs or less than 3000 base pairs. We also identified the full AtNHX3 sequence (Figure 1(d)). A fourth sequence, AtNHX4, was also identified (figure 1(e)).

The coding regions of the nucleic acid molecules are as follows:

Table 1

Nucleic Acid Molecule	Start Nucleotide	End Nucleotide
AtNHX1	286	1902
AtNHX2	61	1707
AtNHX3	67	1024
AtNHX4	55	813

It will be apparent that these may be varied, for example, by shortening the 5' untranslated region or shortening the nucleic acid molecule so that the end nucleotide is in a different position.

The discussion of the nucleic acid molecules, sequence identity, hybridization and other aspects of nucleic acid molecules included within the scope of the invention is intended to be applicable to either the entire nucleic acid molecules in figures 1(a), (b), (d) and (e) and the coding regions of these molecules, shown in Table 1. One may use the entire molecule in figure 1 or only the coding region. Other possible modifications to the sequence will also be apparent.

Southern Blot Analysis (figure 3) suggests that AtNHX1 is likely present as a single copy gene in *Arabidopsis*. A Northern blot (figure 4) showed that AtNHX polypeptide (particularly AtNHX1) was expressed in all tissues examined (root, shoot (shoot includes leaves and stems), flower, inflorescence stem).

Function of Salt Tolerance Nucleic Acid Molecules

The polypeptides of the invention allow the extrusion of monovalent cations (preferably potassium ions or lithium ions, most preferably sodium ions) from the cytosol, which in this application preferably refers to the transport and accumulation of sodium ions into the vacuoles or into the extracellular space (outside of the cell), thus providing the most

important trait for salt tolerance in plants. Antiport polypeptides from organisms other than plants have shown different specificity for monovalent ions (e.g. D.G. Warnock, A.S. Pollock, "Sodium Proton Exchange in Epithelial Cells", pages 77-90, in S. Grinstein ed. Sodium Proton Exchange, (1987, CRC Press, USA).) TNHX and PNHX transporters will also show different specificity between transporters. The nucleic acid molecules of the invention allow the engineering of salt tolerant plants by transformation of crops with this nucleic acid molecule under the control of constitutively active promoters or under the control of conditionally-inducible promoters. The resulting expression or overexpression of these nucleic acid molecules confers increased salt tolerance in plants grown in soil, solid, semi-solid medium or hydroponically.

The PNHX Nucleic Acid Molecule and Polypeptide is Conserved in Plants

Sequence Identity

This is the first isolation of a nucleic acid molecule encoding a Na^+/H^+ exchanger from plant species. It is widely known amongst those skilled in the art that *Arabidopsis thaliana* is a model plant for many plant species. Nucleic acid sequences having sequence identity to the AtNHX sequences are found in other plants, in particular halophytes such as *Beta Vulgaris* and Atriplex (see Examples 2 and 7). Sequences from *Arabidopsis thaliana* and other plants are collectively referred to as "PNHX" nucleic acid sequences and polypeptides. We isolate PNHX nucleic acid molecules from plants having nucleic acid molecules that are similar to those in *Arabidopsis thaliana*, such as beet, tomato, rice, cucumber, radish and other plants as in Table 5 and using techniques described in this application. The invention includes methods of isolating these nucleic acid molecules and polypeptides as well as methods of using these nucleic acid molecules and polypeptides according to the methods described in this application, for example those used with respect to AtNHX.

Table 2 below shows several sequences with sequence identity and sequence similarity to the AtNHX polypeptides. Where polypeptides are shown, a suitable corresponding DNA encoding the polypeptide will be apparent. These sequences code for polypeptides similar to portions of AtNHX polypeptides. The sequences in Table 2 are useful to make probes to identity full length sequences or fragments (from the listed species or other species). One skilled in the art would be able to design a probe based on a polypeptide or peptide fragment. The invention includes nucleic acid molecules of about:

10 to 50 nucleotides, 50 to 200 nucleotides, 200 to 500 nucleotides, 500 to 1000 nucleotides, 1000 to 1500 nucleotides, 1500 to 1700 nucleotides, 1700 to 2000 nucleotides, 2000 to 2500 nucleotides or at least 2500 nucleotides and which include all or part of the sequences (or corresponding nucleic acid molecule) in Table 2. The invention also includes peptides and polypeptides of about: 10 to 50 amino acids, 50 to 200 amino acids, 200 to 500 amino acids, 500 to 750 amino acids or at least 750 amino acids which encode all or part of the polypeptides in Table 2 (wherein the polypeptide is produced according to a reading frame aligned with an AtNHX polypeptide). Possible modifications to these sequences will also be apparent. The polypeptide and nucleic acid molecules are also useful in research experiments or in bioinformatics to locate other sequences. The nucleic acid molecules and polypeptides preferably provide Na^+/H^+ transporter activity and are capable of moving monovalent cations from the cytosol of the cell into vacuoles or the extracellular space (in this application, extracellular space refers to the space outside a cell in an organism or the space outside a cultured cell).

Table 2

Organism	GenBank Accession No.
Yeast (S. pombe) (Fig. 8(a))	3850064
Yeast (Saccharomyces cervisae) (Fig. 8(b))	927695
Rice EST (Fig. 8(c))	C 91832
Rice EST (Fig. 8(d))	C 91861
Rice EST (Fig. 8(e))	AV032544
Medicago Trunculata EST (Fig. 8(f))	AA660573
Hordeum Vulgare STS (Fig. 8g))	L 44032

As shown in Table 3 below, many nucleic acid molecules identified in *Arabidopsis thaliana* have striking DNA sequence similarity to nucleic acid molecules encoding the homologous polypeptide in other plant species. Using the techniques described in this application and others known in the art, it will be apparent that the nucleic acid molecule encoding the homologous Na^+/H^+ exchanger in other plant species including, but not limited to plants of agricultural and commercial interest, will have DNA sequence identity (homology) at least about > 17%, >20%, >25%, >35% to a DNA sequence shown in figure 1 or 5 (or a partial sequence thereof). Some plants species may have DNA with a sequence identity (homology) at least about: >50%, >60%, >70%, >80% or >90% more

preferably at least about >95%, >99% or >99.5%, to a DNA sequence in figure 1 or 5 (or a partial sequence thereof). The invention also includes modified nucleic acid molecules from plants other than *Arabidopsis thaliana* which have sequence identity at least about: > 17%, >20%, >25%, >35%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99% or >99.5%, to an AtNHX sequence in figure 1 or 5 (or a partial sequence thereof). Modified nucleic acid molecules are discussed below. Preferably about 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified. Sequence identity is most preferably calculated as the number of identical amino acid residues expressed as a percentage of the length of the shorter of the two sequences in a pairwise alignment. The pairwise alignment is constructed preferably using the Clustal W program preferably using the following parameter settings: fixed gap penalty=10, floating gap penalty=10, protein weight matrix=BLOSUM62. For example, if a nucleotide sequence (called "Sequence A") has 90% identity to a portion of the nucleotide sequence in Figure 1(a), then Sequence A will be identical to the referenced portion of the nucleotide sequence in Figure 8, except that Sequence A may include up to 10 point mutations, such as substitutions with other nucleotides, per each 100 nucleotides of the referenced portion of the nucleotide sequence in Figure 8. Polypeptides having sequence identity may be similarly identified.

The invention also includes nucleic acid molecules encoding polypeptides having sequence similarity taking into account conservative amino acid substitutions. Sequence similarity (and preferred percentages) are discussed below.

It will be apparent that nucleic acid molecule encoding the homologous Na^+/H^+ exchanger in other species (preferably plants) including, but not limited to plants of agricultural and commercial interest, will hybridize to all or part of a sequence in figure 1 or 5 (or a partial sequence thereof) under low, moderate (also called intermediate conditions) or high stringency conditions. Preferred hybridization conditions are described below.

The invention includes the nucleic acid molecules from other plants as well as methods of obtaining the nucleic acid molecules by, for example, screening a cDNA library or other DNA collection with a probe of the invention (such as a probe comprising at least about: 10 or preferably at least 15 or 30 nucleotides of AtNHX1, AtNHX2, AtNHX3 or AtNHX4 or a sequence in figure 5) and detecting the presence of a TNHX or PNHX nucleic acid molecule. Another method involves comparing the AtNHX sequences (eg in figure 1 or 5) to other sequences, for example using bioinformatics techniques such as database

searches or alignment strategies, and detecting the presence of a TNHX or PNHX nucleic acid molecule or polypeptide. The invention includes the nucleic acid molecule and/or polypeptide obtained according to the methods of the invention. The invention also includes methods of using the nucleic acid molecules, for example to make probes, in research experiments or to transform host cells or make transgenic plants. These methods are as described below.

The polypeptides encoded by the homologous TNHX or PNHX nucleic acid molecules in other species will have amino acid sequence identity. The preferred percentage of sequence identity for sequences of the invention includes sequences having identity of at least about: 30% to AtNHX1, 31% to AtNHX2, 36% to AtNHX3, and 36% to AtNHX4. Sequence identity may be at least about: >20%, >25%, >28%, >30%, >35%, >40%, >50% to an amino acid sequence shown in figure 1 or 5 (or a partial sequence thereof). Some polypeptides may have a sequence identity of at least about: >60%, >70%, >80% or >90%, more preferably at least about: >95%, >99% or >99.5% to an amino acid sequence in figure 1 or 5 (or a partial sequence thereof). Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the Clustal W program. The invention also includes modified polypeptides from plants which have sequence identity at least about: >20%, >25%, >28%, >30%, >35%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99% or >99.5%, to an AtNHX sequence in figure 1 or 5 (or a partial sequence thereof). Modified polypeptides molecules are discussed below. Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

Table 3

Plant Vacuolar H^+-PPiase (vacuolar pyrophosphatase)

	Polypeptide	DNA
Arabidopsis (Accession # 282878)	100%	100%
Beet (Accession # 485742)	88.7%	72.8%
Tobacco (Accession # 1076627)	89.9%	68.4%
Rice (Accession # 1747296)	85%	70.4%

Tonoplast Intrinsic Polypeptide (water channel)

	Polypeptide	DNA
Arabidopsis (Accession # X63551)	100%	100%
Curcubita (Cucumber) (Accession # D45078)	66.5%	39.1%
Raphanus (radish) (Accession # D84669)	56.7%	37.4%
Helianthus (Accession # X95951)	50.4%	35.2%

High Affinity Ammonium Transporter

	Polypeptide	DNA
Arabidopsis (Accession # X75879)	100%	100%
Tomato (Accession # X95098)	73.5%	62.9%
Rice (Accession # AF001505)	66.6%	58.1%

Nucleic Acid Molecules and Polypeptides Similar to AtNHX

Those skilled in the art will recognize that the nucleic acid molecule sequences in figure 1(a), (b), (d) and (e) are not the only sequences which may be used to provide increased salt tolerance in plants. The genetic code is degenerate so other nucleic acid molecules which encode a polypeptide identical to an amino acid sequence in figure 1(a), (b), (d) or (e) may also be used. The sequence of the other nucleic acid molecules of this invention may also be varied without changing the polypeptide encoded by the sequence. Consequently, the nucleic acid molecule constructs described below and in the accompanying examples for the preferred nucleic acid molecules, vectors, and transformants of the invention are merely illustrative and are not intended to limit the scope of the invention.

The sequences of the invention can be prepared according to numerous techniques. The invention is not limited to any particular preparation means. For example, the nucleic

acid molecules of the invention can be produced by cDNA cloning, genomic cloning, DNA synthesis, polymerase chain reaction (PCR) technology, or a combination of these approaches ([31] or Current Protocols in Molecular Biology (F. M. Ausbel et al., 1989).). Sequences may be synthesized using well known methods and equipment, such as automated synthesizers. Nucleic acid molecules may be amplified by the polymerase chain reaction. Polypeptides may, for example, be synthesized or produced recombinantly.

Sequence Identity

The invention includes modified nucleic acid molecules with a sequence identity at least about: >17%, >20%, >30%, >40%, >50%, >60%, >70%, >80% or >90% more preferably at least about >95%, >99% or >99.5%, to a DNA sequence in figure 1 or 5 (or a partial sequence thereof). Preferably about 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified. Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the Clustal W program. For example, if a nucleotide sequence (called "Sequence A") has 90% identity to a portion of the nucleotide sequence in Figure 1(a), then Sequence A will be identical to the referenced portion of the nucleotide sequence in Figure 1, except that Sequence A may include up to 10 point mutations, such as deletions or substitutions with other nucleotides, per each 100 nucleotide of the referenced portion of the nucleotide sequence in Figure 1. Nucleotide sequences functionally equivalent to the PNHX or AtNHX sequences can occur in a variety of forms as described below. Polypeptides having sequence identity may be similarly identified.

The polypeptides encoded by the homologous NHX, PHX Na^+/H^+ exchange nucleic acid molecule in other species will have amino acid sequence identity (also known as homology) at least about: >20%, >25%, >28%, >30%, >40% or >50% to an amino acid sequence shown in figure 1 or 5 (or a partial sequence thereof). Some plants species may have polypeptides with a sequence identity (homology) of at least about: >60%, >70%, >80% or >90%, more preferably at least about: >95%, >99% or >99.5% to all or part of an amino acid sequence in figure 1 or 5 (or a partial sequence thereof). Identity is calculated according to methods known in the art. Sequence identity is most preferably assessed by the Clustal W program. Preferably about: 1, 2, 3, 4, 5, 6 to 10, 10 to 25, 26 to 50 or 51 to 100, or 101 to 250 nucleotides or amino acids are modified.

The invention includes nucleic acid molecules with mutations that cause an amino

acid change in a portion of the polypeptide not involved in providing salt tolerance and ion transport or an amino acid change in a portion of the polypeptide involved in providing salt tolerance so that the mutation increases or decreases the activity of the polypeptide.

Hybridization

Other functional equivalent forms of the *AtNHX* nucleic acid molecules encoding nucleic acids can be isolated using conventional DNA-DNA or DNA-RNA hybridization techniques. These nucleic acid molecules and the *AtNHX* sequences can be modified without significantly affecting their activity.

The present invention also includes nucleic acid molecules that hybridize to one or more of the sequences in figure 1 or 5 (or a partial sequence thereof) or their complementary sequences, and that encode expression for peptides or polypeptides exhibiting substantially equivalent activity as that of an AtNHX polypeptide produced by the DNA in figure 1 or their variants. Such nucleic acid molecules preferably hybridize to the sequences under low, moderate (intermediate), or high stringency conditions. (see Sambrook et al. (Most recent edition) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Preferable hybridization conditions are about those in Table 4.

The present invention also includes nucleic acid molecules from any source, whether modified or not, that hybridize to genomic DNA, cDNA, or synthetic DNA molecules that encode the amino acid sequence of an AtNHX polypeptide, or genetically degenerate forms, under salt and temperature conditions equivalent to those described in this application, and that code for a peptide, polypeptide or polypeptide that has Na^+/H^+ transporter activity. Preferably the polypeptide has the same or similar activity as that of an AtNHX polypeptide. The nucleic acid molecules may encode TNHX or PNHX polypeptides. A nucleic acid molecule described above is considered to be functionally equivalent to an AtNHX nucleic acid molecule (and thereby having Na^+/H^+ transporter activity) of the present invention if the polypeptide produced by the nucleic acid molecule displays the following characteristics: the polypeptide mediates the proton-dependent sodium transport and sodium-dependent proton transport in intact cells, isolated organelles and purified membrane vesicles. These sodium/proton movements should be higher (preferably at least about 50% higher and most preferably at least about 100% higher) than the proton movements observed in the presence of a background of potassium ions and/or other

monovalent cations (i.e. rubidium, cesium, etc., but most preferably not lithium) (13,14).

The invention also includes nucleic acid molecules and polypeptides having sequence similarity taking into account conservative amino acid substitutions. Sequence similarity (and preferred percentages) are discussed below.

Modifications to Nucleic Acid Molecule or Polypeptide Sequence

Changes in the nucleotide sequence which result in production of a chemically equivalent or chemically similar amino acid sequences are included within the scope of the invention. Variants of the polypeptides of the invention may occur naturally, for example, by mutation, or may be made, for example, with polypeptide engineering techniques such as site directed mutagenesis, which are well known in the art for substitution of amino acids. For example, a hydrophobic residue, such as glycine can be substituted for another hydrophobic residue such as alanine. An alanine residue may be substituted with a more hydrophobic residue such as leucine, valine or isoleucine. A negatively charged amino acid such as aspartic acid may be substituted for glutamic acid. A positively charged amino acid such as lysine may be substituted for another positively charged amino acid such as arginine.

Therefore, the invention includes polypeptides having conservative changes or substitutions in amino acid sequences. Conservative substitutions insert one or more amino acids which have similar chemical properties as the replaced amino acids. The invention includes sequences where conservative substitutions are made that do not destroy Na^+/H^+ transporter activity of the transporter polypeptide. The preferred percentage of sequence similarity for sequences of the invention includes sequences having at least about: 48% similarity to AtNHX1, 48% similarity to AtNHX2, 56% similarity to AtNHX3, and 56% similarity to AtNHX4. The similarity may also be at least about: 60% similarity, 75% similarity, 80% similarity, 90% similarity, 95% similarity, 97% similarity, 98% similarity, 99% similarity, or more preferably at least about 99.5% similarity, wherein the polypeptide Na^+/H^+ has transporter activity. The invention also includes nucleic acid molecules encoding polypeptides, with the polypeptides having at least about: at least about: 48% similarity to AtNHX1, 48% similarity to AtNHX2, 56% similarity to AtNHX3, and 56% similarity to AtNHX4. The similarity may also be at least about: 60% similarity, 75% similarity, 80% similarity, 90% similarity, 95% similarity, 97% similarity, 98% similarity, 99% similarity, or more preferably at least about 99.5% similarity, wherein the polypeptide

Na^+/H^+ has transporter activity, to an amino acid sequence in figure 1 or 5 (or a partial sequence thereof) considering conservative amino acid changes, wherein the polypeptide has Na^+/H^+ transporter activity. Sequence similarity is preferably calculated as the number of similar amino acids in a pairwise alignment expressed as a percentage of the shorter of the two sequences in the alignment. The pairwise alignment is preferably constructed using the Clustal W program, using the following parameter settings: fixed gap penalty=10, floating gap penalty=10, protein weight matrix=BLOSUM62. Similar amino acids in a pairwise alignment are those pairs of amino acids which have positive alignment scores defined in the preferred protein weight matrix (BLOSUM62). The protein weight matrix BLOSUM62 is considered appropriate for the comparisons described here by those skilled in the art of bioinformatics. (The reference for the clustal w program (algorithm) is Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22:4673-4680; and the reference for BLOSUM62 scoring matrix is Henikoff, S. and Henikoff, J.G. (1993) Performance evaluation of amino acid substitution matrices. Proteins, 7:49-61.)

Polypeptides comprising one or more d-amino acids are contemplated within the invention. Also contemplated are polypeptides where one or more amino acids are acetylated at the N-terminus. Those of skill in the art recognize that a variety of techniques are available for constructing polypeptide mimetics with the same or similar desired biological activity (Na^+/H^+ transporter activity) as the corresponding polypeptide compound of the invention but with more favorable activity than the polypeptide with respect to solubility, stability, and/or susceptibility to hydrolysis and proteolysis. See, for example, Morgan and Gainor, *Ann. Rep. Med. Chem.*, 24:243-252 (1989). Examples of polypeptide mimetics are described in U.S. Patent Nos. 5,643,873. Other patents describing how to make and use mimetics include, for example in, 5,786,322, 5,767,075, 5,763,571, 5,753,226, 5,683,983, 5,677,280, 5,672,584, 5,668,110, 5,654,276, 5,643,873. Mimetics of the polypeptides of the invention may also be made according to other techniques known in the art. For example, by treating a polypeptide of the invention with an agent that chemically alters a side group by converting a hydrogen group to another group such as a hydroxy or amino group. Mimetics preferably include sequences that are either entirely made of amino acids or sequences that are hybrids including amino acids and modified amino acids or other organic molecules.

The invention also includes hybrid nucleic acid molecules and polypeptides, for example where a nucleotide sequence from one species of plant is combined with a nucleotide sequence from another sequence of plant, mammal or yeast to produce a fusion polypeptide. The invention includes a fusion protein having at least two components, wherein a first component of the fusion protein comprises a polypeptide of the invention, preferably a full length AtNHX polypeptide. The second component of the fusion protein preferably comprises a tag, for example GST, an epitope tag or an enzyme. The fusion protein may comprise lacZ.

The invention also includes polypeptide fragments of the polypeptides of the invention which may be used to confer salt tolerance if the fragments retain Na^+/H^+ transporter activity. The invention also includes polypeptides fragments of the polypeptides of the invention which may be used as a research tool to characterize the polypeptide or its activity. Such polypeptides preferably consist of at least 5 amino acids. In preferred embodiments, they may consist of 6 to 10, 11 to 15, 16 to 25, 26 to 50, 51 to 75,76 to 100 or 101 to 250 amino acids of the polypeptides of the invention (or longer amino acid sequences). The fragments preferably have sodium/proton transporter activity. Fragments may include sequences with one or more amino acids removed, for example, C-terminus amino acids in an AtNHX sequence.

The invention also includes a composition comprising all or part of an isolated TNHX or PNHX (preferably AtNHX) nucleic acid molecule of the invention and a carrier, preferably in a composition for plant transformation . The invention also includes a composition comprising an isolated TNHX or PNHX polypeptide (preferably AtNHX) and a carrier, preferably for studying polypeptide activity.

Recombinant Nucleic Acid Molecules

The invention also includes recombinant nucleic acid molecules comprising a nucleic acid molecule of the invention and a promoter sequence, operatively linked so that the promoter enhances transcription of the nucleic acid molecule in a host cell (the nucleic acid molecules of the invention may be used in an isolated native gene or a chimeric gene (for example, where a nucleic acid molecule coding region is connected to one or more heterologous sequences to form a gene). The promoter sequence is preferably a constitutive promoter sequence or an inducible promoter sequence, operatively linked so that the promoter enhances transcription of the DNA molecule in a host cell. The promoter

may be of a type not naturally associated with the cell. Transcription is enhanced with promoters known in the art such as the "Super-promoter" [20] or the 35S promoter of cauliflower mosaic virus [21].

Inducible promoters are also used. These include:

a) drought- and ABA-inducible promoters which may include ABA-responsive elements [22,23]

b) heat shock-inducible promoters which may contain HSEs (heat shock elements) as well as CCAAT box sequences [24]

c) salt-inducible promoters which may include AT and PR elements [25]

d) Copper-inducible promoter that includes ACE1 binding sites [26]

e) steroid-inducible promoter that includes the glucocorticoid response element along with an expression vector coding for a mammalian steroid receptor [27].

In addition, tissue specific expression is achieved with the use of tissue-specific promoters such as, the Fd (Ferredoxin) promoter that mediates high levels of expression in green leaves [28] and peroxidase promoter for root-specific expression [29]. These promoters vary in their transcription initiation rate and/or efficiency.

A recombinant nucleic acid molecule for conferring salt tolerance may also contain suitable transcriptional or translational regulatory elements. Suitable regulatory elements may be derived from a variety of sources, and they may be readily selected by one with ordinary skill in the art. Examples of regulatory elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the vector employed, other genetic elements, such as selectable markers, may be incorporated into the recombinant molecule. Markers facilitate the selection of a transformed host cell. Such markers include genes associated with temperature sensitivity, drug resistance, or enzymes associated with phenotypic characteristics of the host organisms.

Nucleic acid molecule expression levels are controlled with a transcription initiation region that regulates transcription of the nucleic acid molecule or nucleic acid molecule fragment of interest in a plant, bacterial or yeast cell. The transcription initiation region may be part of the construct or the expression vector. The transcription initiation domain or promoter includes an RNA polymerase binding site and an mRNA initiation site. Other

regulatory regions that may be used include an enhancer domain and a termination region. The regulatory elements described above may be from animal, plant, yeast, bacterial, fungal, viral or other sources, including synthetically produced elements and mutated elements.

Methods of modifying DNA and polypeptides, preparing recombinant nucleic acid molecules and vectors, transformation of cells, expression of polypeptides are known in the art. For guidance, one may consult the following US patent nos. 5,840,537, 5,850,025, 5,858,719, 5,710,018, 5,792,851, 5,851,788, 5,759,788, 5,840,530, 5,789,202, 5,871,983, 5,821,096, 5,876,991, *5,422,108,* 5,612,191, 5,804,693, 5,847,258, 5,880,328, 5,767,369, 5,756,684, 5,750,652, 5,824,864, 5,763,211, 5,767,375, or 5,750,848. Many of these patents also provide guidance with respect to experimental assays, probes and antibodies, transformation of host cells and regeneration of plants, which are described below. These patents, like all other patents, publications (such as articles and Genbank publications) in this application, are incorporated by reference in their entirety.

Host Cells Including a Salt Tolerance Nucleic Acid Molecule

In a preferred embodiment of the invention, a plant or yeast cell is transformed with a nucleic acid molecule of the invention or a fragment of a nucleic acid molecule and inserted in a vector.

Another embodiment of the invention relates to a method of transforming a host cell with a nucleic acid molecule of the invention or a fragment of a nucleic acid molecule, inserted in a vector. The invention also includes a vector comprising a nucleic acid molecule of the invention. The TNHX, PNHX and AtNHX nucleic acid molecules can be cloned into a variety of vectors by means that are well known in the art. The recombinant nucleic acid molecule may be inserted at a site in the vector created by restriction enzymes. A number of suitable vectors may be used, including cosmids, plasmids, bacteriophage, baculoviruses and viruses. Suitable vectors are capable of reproducing themselves and transforming a host cell. The invention also relates to a method of expressing polypeptides in the host cells. A nucleic acid molecule of the invention may be used to transform virtually any type of plant, including both monocots and dicots. The expression host may be any cell capable of expressing TNHX, PNHX, such as a cell selected from the group consisting of a seed (where appropriate), plant cell, bacterium, yeast, fungus, protozoa, algae, animal and animal cell.

Levels of nucleic acid molecule expression may be controlled with nucleic acid

molecules or nucleic acid molecule fragments that code for anti-sense RNA inserted in the vectors described above.

Agrobacterium tumefaciens-mediated transformation, particle-bombardment-mediated transformation, direct uptake, microinjection, coprecipitation and electroporation-mediated nucleic acid molecule transfer are useful to transfer a Na^+/H^+ transporter nucleic acid molecule into seeds (where appropriate) or host cells, preferably plant cells, depending upon the plant species. The invention also includes a method for constructing a host cell capable of expressing a nucleic acid molecule of the invention, the method comprising introducing into said host cell a vector of the invention. The genome of the host cell may or may not also include a functional TNHX or PNHX gene. The invention also includes a method for expressing a TNHX or PNHX transporter polypeptide in the host cell or a plant, plant part, seed or plant cell of the invention, the method comprising culturing the host cell under conditions suitable for gene expression. The method preferably also includes recovering the expressed polypeptide from the culture.

The invention includes the host cell comprising the recombinant nucleic acid molecule and vector as well as progeny of the cell. Preferred host cells are fungal cells, yeast cells, bacterial cells, mammalian cells, bird cells, reptile cells, amphibious cells, microorganism cells and plant cells. Host cells may be cultured in conventional nutrient media. The media may be modified as appropriate for inducing promoters, amplifying genes or selecting transformants. The culture conditions, such as temperature, composition and pH will be apparent. After transformation, transformants may be identified on the basis of a selectable phenotype. A selectable phenotype can be conferred by a selectable marker in the vector.

Transgenic Plants and Seeds

Plant cells are useful to produce tissue cultures, seeds or whole plants. The invention includes a plant, plant part, seed, or progeny thereof including a host cell transformed with a PNHX nucleic acid molecule. The plant part is preferably a leaf, a stem, a flower, a root, a seed or a tuber.

The invention includes a transformed (transgenic) plant having increased salt tolerance, the transformed plant containing a nucleic acid molecule sequence encoding for Na^+/H^+ transporter polypeptide activity and the nucleic acid molecule sequence having been introduced into the plant by transformation under conditions whereby the transformed

plant expresses a Na^+/H^+ transporter in active form.

The methods and reagents for producing mature plants from cells are known in the art. The invention includes a method of producing a genetically transformed plant which expresses PNHX or TNHX polypeptide by regenerating a genetically transformed plant from the plant cell, seed or plant part of the invention. The invention also includes the transgenic plant produced according to the method. Alternatively, a plant may be transformed with a vector of the invention.

The invention also includes a method of preparing a plant with increased salt tolerance, the method comprising transforming the plant with a nucleic acid molecule which encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide encoding a Na^+/H^+ transporter polypeptide capable of increasing salt tolerance in a cell, and recovering the transformed plant with increased salt tolerance. The invention also includes a method of preparing a plant with increased salt tolerance, the method comprising transforming a plant cell with a nucleic acid molecule which encodes a TNHX transporter polypeptide, a PNHX transporter polypeptide or a polypeptide encoding a Na+/H+ transporter polypeptide capable of increasing salt tolerance in a cell, and producing the transformed plant with increased salt tolerance.

Overexpression of Na^+/H^+ exchangers leads to an improved ability of the transgenic plants to uptake more monovalent cations from the growth media (soil) leading to an increased or enhanced tissue expansion. Figure 7 shows that transformed plants have grown larger even where no NaCl is added to soil. Therefore, the invention also relates to methods of producing or growing plants with increased tissue expansion (this could be manifested as enhanced size, growth or growth potential and may appear as increased or enhanced root, crown, shoot, stem, leaf, flower size or abundance in comparison to a wild type plant). The methods of preparing plants that have increased tissue expansion are the same as the methods for preparing a plant with increased salt tolerance described in this application (or the methods are easily adapted, to the extent that there is a difference in the methods).

The plants whose cells may be transformed with a nucleic acid molecule of this invention and used to produce transgenic plants include, but are not limited to the following:

Target plants:

Group I (transformable preferably via *Agrobacterium tumefaciens*)

Arabidopsis
Potato
Tomato
Brassica
Cotton
Sunflower
Strawberries
Spinach
Lettuce
Rice

Group II (transformable preferably via biolistic particle delivery systems (particle bombardment)

Soybean
Rice
Corn
Wheat
Rye
Barley
Atriplex
Salicornia

The nucleic acid molecule may also be used with other plants such as oat, barley, hops, sorgum, alfalfa, sunflower, alfalfa, beet, pepper, tobacco, melon, squash, pea, cacao, hemp, coffee plants and grape vines. Trees may also be transformed with the nucleic acid molecule. Such trees include, but are not limited to maple, birch, pine, oak and poplar. Decorative flowering plants such as carnations and roses may also be transformed with the nucleic acid molecule of the invention. Plants bearing nuts such as peanuts may also be transformed with the salt tolerance nucleic acid molecule. A list of preferable plants is in Table 5.

In a preferred embodiment of the invention, plant tissue cells or cultures which demonstrate salt tolerance are selected and plants which are salt tolerant are regenerated from these cultures. Methods of regeneration will be apparent to those skilled in the art (see Examples below, also). These plants may be reproduced, for example by cross pollination with a plant that is salt tolerant or a plant that is not salt tolerant. If the plants are self-pollinated, homozygous salt tolerant progeny may be identified from the seeds of these

plants, for example by growing the seeds in a saline environment, using genetic markers or using an assay for salt tolerance. Seeds obtained from the mature plants resulting from these crossings may be planted, grown to sexual maturity and cross-pollinated or self-pollinated.

The nucleic acid molecule is also incorporated in some plant species by breeding methods such as back crossing to create plants homozygous for the salt resistance nucleic acid molecule.

A plant line homozygous for the salt tolerance nucleic acid molecule may be used as either a male or female parent in a cross with a plant line lacking the salt tolerance nucleic acid molecule to produce a hybrid plant line which is uniformly heterozygous for the nucleic acid molecule. Crosses between plant lines homozygous for the salt resistance nucleic acid molecule are used to generate hybrid seed homozygous for the resistance nucleic acid molecule.

The nucleic acid molecule of the invention may also be used as a marker in transformation experiments with plants. A salt sensitive plant may be transformed with a salt tolerance nucleic acid molecule and a nucleic acid molecule of interest which are linked. Plants transformed with the nucleic acid molecule of interest will display improved growth in a saline environment relative to the non-transformed plants.

Fragments/Probes

Preferable fragments (fragments are also referred to as polypeptide fragments or peptide fragments) include 10 to 50, 50 to 100, 100 to 250, 250 to 500, 500 to 1000, 1000 to 1500, or 1500 or more nucleotides of a nucleic acid molecule of the invention. A fragment may be generated by removing a single nucleotide from a sequence in figure 1 or 5 (or a partial sequence thereof). Fragments may or may not have Na^+/H^+ transporter activity.

The nucleic acid molecules of the invention (including a fragment of a sequence in figure 1 or 5 (or a partial sequence thereof) (such as [SEQ ID NO:1], [SEQ ID NO:3], [SEQ ID NO:5] or [SEQ ID NO:7]) can be used as probes to detect nucleic acid molecules according to techniques known in the art (for example, see US patent nos. 5,792,851 and 5,851,788). The probes may be used to detect nucleic acid molecules that encode polypeptides similar to the polypeptides of the invention. For example, a probe having at least about 10 bases will hybridize to similar sequences under stringent hybridization

conditions (Sambrook et al. 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor).

The invention includes oligonucleotide probes made from the AtNHX sequences described in this application or other nucleotide sequences of the invention. The probes may be about 10 to 30 or 15 to 30 nucleotides in length and are preferably at least 30 or more nucleotides. A preferred probe is 5'-TTCTTCATATATCTTTTGCCACCC-3' (coding for the amiloride binding domain) or at least about 10 or 15 nucleotides of this sequence. The invention also includes an oligonucleotide including at least 30 consecutive nucleotides of an AtNHX molecule in Figure 1 or 5 (or a partial sequence thereof). The probes are useful to identify nucleic acids encoding AtNHX, polypeptides and proteins other than those described in the application, as well as peptides, polypeptides, and proteins have Na^+/H^+ transporter activity and preferably functionally equivalent to AtNHX. The oligonucleotide probes are capable of hybridizing to one or more of the sequences shown in Figure 1 or 5 (or a partial sequence thereof) or the other sequences of the invention under low, moderate or high stringency hybridization conditions. A nucleotide sequence encoding a polypeptide of the invention may be isolated from other organisms by screening a library under low, moderate or high stringency hybridization conditions with a detectable probe (e.g. a labeled probe). The activity of the polypeptide encoded by the nucleotide sequence may be assessed by cloning and expression of the DNA. After the expression product is isolated, the polypeptide is assayed for Na^+/H^+ transporter activity as described in this application.

Functionally equivalent AtNHX, TNHX or PNHX nucleic acid molecules from other cells, or equivalent AtNHX, TNHX or PNHX -encoding cDNAs or synthetic DNAs, can also be isolated by amplification using Polymerase Chain Reaction (PCR) methods. Oligonucleotide primers, including degenerate primers, based on the amino acid sequence of the sequences in Figures 1 or 5 (or a partial sequence therof) can be prepared and used in conjunction with PCR technology employing reverse transcriptase to amplify functionally equivalent DNAs from genomic or cDNA libraries of other organisms. Alternatively, the oligonucleotides, including degenerate nucleotides, can be used as probes to screen cDNA libraries.

Thus, the invention includes an oligonucleotide probe comprising all or part of a nucleic acid in figure 1 or 5 (or a partial sequence thereof), or a complementary strand thereof. The probe is preferably labeled with a detectable marker. The invention also includes an oligonucleotide comprising at least 10, 15 or 30 nucleotides capable of

specifically hybridizing with a sequence of nucleic acids of the nucleotide sequence set forth in figure 1 or 5 (or a partial sequence thereof). The invention also includes a single strand DNA primer for amplification of PNHX nucleic acid, wherein the primer is selected from a nucleic acid sequence derived from a nucleic acid sequence in figure 1 or 5 (or a partial sequence thereof).

The invention also includes a method for identifying nucleic acid molecules encoding a TNHX, PNHX or AtNHX polypeptide. Techniques for performing the methods are described in, for example, US Patent Nos. 5,851,788 and 5,858,719. A preferred method includes contacting a sample containing nucleic acids with an oligonucleotide, wherein said contacting is effected under low, moderate or high stringency hybridization conditions, and identifying nucleic acids which hybridize thereto. Hybridization forms a hybridization complex. The presence of a complex correlates with the presence of a nucleic acid molecule encoding TNHX, plant PNHX polypeptide or AtNHX in the sample. In a preferred method, the nucleic acid molecules are amplified by the polymerase chain reaction prior to hybridization.

KITS

The invention also includes a kit for conferring increased salt tolerance to a plant or a host cell including a nucleic acid molecule of the invention (preferably in a composition fo the invention) and preferably reagents for transforming the plant or host cell.

The invention also includes a kit for detecting the presence of a TNHX or a PNHX nucleic acid molecule, comprising at least one oligonucleotide of the invention. Kits may be prepared according to known techniques, for example, see patent nos. 5,851,788 and 5,750,653.

Antibodies

The invention includes an isolated antibody immunoreactive with a polypeptide of the invention (see Example 1). The antibody may be labeled with a detectable marker or unlabeled. The antibody is preferably a monoclonal antibody or a polyclonal antibody. TNHX, PNHX or AtNHX antibodies can be employed to screen organisms containing TNHX, PNHX or AtNHX polypeptides. The antibodies are also valuable for immuno-purification of polypeptides from crude extracts.

The isolated antibody is preferably specifically reactive with a TNHX or PNHX transporter, preferably an AtNHX transporter. The transporter is preferably encoded by a nucleic acid molecule in figure 1 (or molecules that hybridize to a molecule in figure 1 under low, moderate or high stringency hybridization conditions or molecules having at least about: 17%, at least 20%, at least 25%, or at least 35% sequence identity (or the other preferred percentages of identity or sequence similarity described above) to a molecule in figure 1 or 5 (or a partial sequence thereof). The transporter is preferably a polypeptide in figure 1 (or polypeptides having at least about: 28%, 35% sequence identity (or the other preferred percentages of identity or sequence similarity described above) to a polypeptide in figure 1 or 5 (or a partial sequence thereof). The antibody preferably does not cross-react with other transporter polypeptides. The antibody is preferably specifically reactive with a polypeptide having an amino acid sequence encoded by a nucleic acid molecule set forth in figure 1 or 5 (or a partial sequence thereof).

Examples of the preparation and use of antibodies are provided in US Patent Nos. 5,792,851 and 5,759,788. For other examples of methods of the preparation and uses of monoclonal antibodies, see US Patent Nos. 5,688,681, 5,688,657, 5,683,693, 5,667,781, 5,665,356, 5,591,628, 5,510,241, 5,503,987, 5,501,988, 5,500,345 and 5,496,705. Examples of the preparation and uses of polyclonal antibodies are disclosed in US Patent Nos. 5,512,282, 4,828,985, 5,225,331 and 5,124,147.

The invention also includes methods of using the antibodies. For example, the invention includes a method for detecting the presence of TNHX, PNHX or AtNHX transporter polypeptide, by: a) contacting a sample containing one or more polypeptides with an antibody of the invention under conditions suitable for the binding of the antibody to polypeptides with which it is specifically reactive; b) separating unbound polypeptides from the antibody; and c) detecting antibody which remains bound to one or more of the polypeptides in the sample.

Research Tool

Cell cultures, seeds, plants and plant parts transformed with a nucleic acid molecule of the invention are useful as research tools. For example, one may obtain a plant cell (or a cell line, such as an immortalized cell culture or a primary cell culture) that does not express AtNHX1, insert an AtNHX1 nucleic acid molecule in the cell, and assess the level of AtNHX1 expression and activity. Alternatively, PNHX nucleic acid molecules may be

overexpressed in a plant that expresses a PNHX nucleic acid molecule. In another example, experimental groups of plants may be transformed with vectors containing different types of PNHX nucleic acid molecules (or PNHX nucleic acid molecules similar to PNHX or fragments of PNHX nucleic acid molecules) to assess the levels of protein produced, its functionality and the phenotype of the plants (for example, phenotype in saline soil). The polypeptides are also useful for *in vitro* analysis of TNHX, PNHX or AtNHX activity or structure. For example, the polypeptides produced can be used for microscopy or X-ray crystallography studies.

The TNHX, PNHX or AtNHX nucleic acid molecules and polypeptides are also useful in assays. Assays are useful for identification and development of compounds to inhibit and/or enhance polypeptide function directly. For example, they are useful in an assay for evaluating whether test compounds are capable of acting as antagonists for PNHX polypeptides by: (a) culturing cells containing: a nucleic acid molecule which expresses PNHX polypeptides (or polypeptides having PNHX or Na+/H+ activity) wherein the culturing is carried out in the presence of: increasing concentrations of at least one test compound whose ability to inhibit transport activity of PNHX polypeptide is sought to be determined, and a fixed concentration of salt; and (b) monitoring in the cells the level of salt transported out of the cytosol as a function of the concentration of the test compound, thereby indicating the ability of the test compound to inhibit PNHX transporter activity. Alternatively, the concentration of the test compound may be fixed and the concentration of salt may be increased.

Another experiment is an assay for evaluating whether test compounds are capable of acting as agonists for PNHX polypeptide characterized by being able to transport salt across a membrane, (or polypeptides having PNHX or Na+/H+ transporter activity) by (a) culturing cells containing: a nucleic acid molecule which expresses PNHX polypeptide or (or polypeptides having PNHX activity) thereof, wherein said culturing is carried out in the presence of: fixed concentrations of at least one test compound whose ability to increase or enhance salt transport activity of PNHX polypeptide is sought to be determined, and an increasing concentration of salt; and (b) monitoring in the cells the level of salt transported out of the cytosol as a function of the concentration of the test compound, thereby indicating the ability of the test compound compound to increase or enhance PNHX polypeptide activity. Alternatively, the concentration of the test compound may be fixed and the concentration of salt may be increased. Suitable assays may be adapted from, for

example, US patent no. 5,851,788. It is apparent that TNHX and AtNHX may also be used in assays.

Bioremediation

Soils containing excessive salt may be unable to grow plants in a manner suitable for agriculture. The invention includes a method for removing salt from a growth medium, comprising growing a plant transformed with a nucleic acid molecule of the invention and expressing a salt tolerance Na^+/H^+ transporter polypeptide in the growth medium for a time period sufficient for the plant root to uptake and accumulate salt in the root or shoot biomass. The growth medium may be a solid medium, semi-solid medium, liquid medium or a combination thereof. It may include soil, sand, sludge, compost, or artificial soil mix. The shoot (leaf or stem) or and root biomass may be harvested. Preferably, a sufficient portion of the shoot biomass is not harvested and is left in the growth media to permit continued plant growth.

Using Exogenous Agents in Combination with a Vector

The nucleic acid molecules of the invention may be used with other nucleic acid molecules that relate to salt tolerance, for example, osmoregulant genes. Host cells or plants may be transformed with these nucleic acid molecules. Osmoregulants are disclosed, for example, in US Patent Nos. 5,563,324 and 5,639, 950.

It will be clear to those skilled in the art that sequences in figure 1(c) and 5(a) and (b) are also useful, for example in preparation of probes or as experimental tools or as antigens to which antibodies may be directed. The following Examples are intended to illustrate and assist in the further understanding of the invention. Particular materials employed, species, conditions and the like are not intended to limit the reasonable scope of the invention.

Example 1

Preparation of polyclonal and monoclonal antibodies.

Hydropathy profiles of the *Arabidopsis* Na^+/H^+ antiport revealed a relatively hydrophilic domain (at the C-terminus) with possible regulatory functions. The C-terminus was sub-cloned into the pGEX – 2TK vector (Pharmacia) to allow the overexpression of the C-terminus polypeptide as a GST-fusion polypeptide in *E. coli*. The fusion polypeptide was purified by glutathione-affinity chromatography and used as an antigen in rabbits to obtain

polyclonal antibodies [30].

Monoclonal antibodies are prepared in mice hybridomas according to established techniques [30] using the C-terminus polypeptide as described above. Polyclonal and monoclonal antibodies raised against other regulatory regions of the *Arabidopsis* Na^+/H^+ antiport are also obtained as described above. The invention includes the antibodies and the hybridoma which secretes the monoclonal antibodies.

Example 2

Identification of homologous nucleic acid molecules from other plant species, preferably salt tolerant species.

Several experimental approaches are used to identify homologous nucleic acid molecules from salt tolerant species. a) We screen cDNA and genomic libraires from sugar beets (a moderate salt-tolerant crop, also known as red beet) under low-stringency conditions with an Arabidopsis Na^+/H^+ antiport cDNA as a probe [31]; b) We apply PCR techniques using degenerate oligonucleotide primers designed according to the conserved regions of the Arabidopsis Na^+/H^+ antiport [32]; c) We screen cDNA expression libraries from different plants (salt-tolerant and salt-sensitive) using antibodies raised against an Arabidopsis Na^+/H^+ antiport [31]. We also use bioinformatics techniques to identify nucleic acid molecules. The invention includes methods of using such a nucleic acid molecule, for example to express a recombinant polypeptide in a transformed cell.

The techniques described above for isolating nucleic acid molecules from *Arabidopsis* and sugar beet are used to isolate a salt tolerance nucleic acid molecule from Atriplex and other plants.

Example 3

Overexpression of the PNHX transporter, preferably *Arabidopsis* transporter (AtNHX).

The Na^+/H^+ antiport is expressed in *Arabidopsis* plants, although the wild type plants show impaired growth at NaCl concentrations higher than 75 mM. The Na^+/H^+ antiport is overexpressed in these plants in order to improve their tolerance to high salt concentrations. A full length cDNA (preferably coding for the AtNHX1 polypeptide (AtNHX2, AtNHX3 or AtNHX4) cloned from an *Arabidopsis thaliana* (Columbia) seedling cDNA library is ligated into a pBINS1 vector [33]. This vector contains a constitutively strong promotor ("super-promotor' [20]). Also, T-DNA vectors (pBECKS) are used [34].

Constructs containing the AtNHX1 cDNA with the full Na^+/H^+ antiport open reading frame in a sense orientation were selected by colony hybridization using the AtNHX1 as a probe and by restriction-digest analysis using BglII restriction endonuclease. These constructs are used to transform *Agrobacterium tumefaciens*, and these transformed *Agrobacterium tumefaciens* are used for transformation of *Arabidopsis* plants. The *Agrobacterium* for inoculation is grown at 28°C in a medium containing 5g/l Bacto Beef Extract, 5g/l Bacto-Peptone, 1g/l Bacto Yeast Extract, 240 mg $MgSO_4$ and 5g/l sucrose. The pH will be adjusted to 7.2 with NaOH.

Arabidopsis seeds are washed and surface-sterilized in 5% (w/v) sodium hypochlorite containing 0.15% (v/v) Tween-20. The seeds are rinsed thoroughly with sterile distilled water. Seed aliquots are dispensed in flasks containing 45 ml of cocultivation medium (MS salts, 100 mM sucrose, 10 mg/l thiamine, 0.5 mg/l pyridoxine, 0.5 mg/l nicotinic acid, 100 mg/l inositol and the pH adjusted to 6.0 with KOH. The flasks are incubated at 22°C under constant rotation (190 rpm) and constant light. After 10-18 h (time needed to break clumps of seeds) 5 ml of log phase of *Agrobacterium* (OD_{600}=0.75) carrying the AtNHX1 construct are added. Twenty-four hours following the inoculation, the seeds are dried by filtration and sown into pre-soaked vermiculite. The flats containing the seeds are irrigated as required with a half-Hoagland solution. The flats are covered with plastic to prevent desiccation and maintained at low artificial illumination. After 3 days the flats are transferred to the greenhouse (the plastic cover removed) under a 16/8 day/night cycle. Supplementary light is provided by high pressure sodium vapor lights. Seven weeks after sowing, the plants are dried thoroughly and the seeds (T2) harvested. Transformation efficiency is estimated by plating 100,000 seeds (approximately 2.5 g of seeds) on agar plates containing 50 mg/l kanamycin in a medium containing 1% (w/v) sucrose, 0.8 (w/v) agar, MS salts and a pH 6.0 adjusted with KOH. The plates are transferred to a growth room at 25°C under continuous light. After 10 days the kanamycin-resistant seedlings are transferred to new growth medium for 2 weeks and then transferred to small pots containing vermiculite. At senescence (8 weeks) the seeds are collected from single plants (T3). These seeds are germinated and used to assess salt tolerance of the transgenic plants.

Example 4

Overexpression of TNHX or PNHX in other plants.

In a preferred method, overexpression of PNHX, preferably AtNHX1, AtNHX2,

AtNHX3 or AtNHX4, in a number of plants (potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, salicornia, and others) is achieved by *Agrobacterium tumefaciens*-based transformation and/or particle bombardment (AtNHX2, AtNHX3, AtNHX4 are also useful in this example). The full length cDNA (coding for the AtNHX1) is ligated into the pBINS1 vector or pBECKS (as described above) and these constructs are used to transform *Agrobacterium tumefaciens* strain LBA4404. *Agrobacterium* used for inoculation is grown as described above. Cultured cells (callus), leaf explants, shoot and root cultures are used as targets for transformation. The targeted tissues are co-cultivated with the bacteria for 1 - 2 days. Afterwards, the tissue is transferred to a growth media containing kanamycin. After one week the tissue is transferred to a regeneration medium containing MS salts, 1% sucrose, 2.5 mg/l 3-benzyladenine, 1 mg/l zeatin, 0.75% agar and kanamycin. Weekly transfers to fresh regeneration media are performed.

In another preferred embodiment, overexpression constructs carrying the *AtNHX1* cDNA are introduced into an electro-competent *Agrobacterium tumefaciens* (LBA4404) by electroporation. The *Agrobacteria* are plated on LB plates containing 50 mg/L kanamycin and grown for ~2 days at 30 ^{o}C to select for bacteria carrying the overexpression constructs. One liter liquid LB+kanamycin (50mg/L) is inoculated with a single *Agrobacterium* colony selected from the LB (kanamycin 50mg/L) plates. The culture is grown to a minimum of OD=1 (600nm) for 2-3 days. The *Agrobacteria* are then pelleted and resuspended in 1L infiltration medium (IM - 0.5XMS salts; 0.5 g/L MES; 5% sucrose; 0.03% Silwet L-77). Flowering *Arabidopsis* plants with primary bolts reaching ~15cm are used for the transformation procedure (T1). Pots of *Arabidopsis* plants are dunked into the IM solution containing the *Agrobacteria* and left submerged for 2-6 minutes. The same procedure can be repeated after 8-12 days on the same plants. Plants are allowed to senesce, the plants are dried thoroughly and the seeds harvested. Seeds are plated on agar plates containing 25 mg/L kanamycin in a medium containing MS salts, 0.8% (w/v) agar adjusted to pH 6.0 with KOH. The plates are transferred to a growth room at 25 ^{o}C under continuous light. After 10 days the kanamycin-resistant seedlings (T2) are transferred to small pots containing vermiculite. At senescence (~8 weeks) the seeds are collected from single plants and plated on agar plates containing MS salts and 25mg/L kanamycin. After 10 days the kanamycin-resistant seedlings (T3) are transferred to small pots containing vermiculite. Seeds produced by these plants are germinated and used to assess

salt tolerance of the transgenic plants.A biolistic particle delivery system (particle bombardment) is also used for the overexpression of NHX (AtNHX1, AtNHX2, AtNHX3 or AtNHX4 are useful for this example). Constructs made using a plasmid vector preferably carrying a constitutive promoter, the AtNHX1 open reading frame in a sense orientation and a NOS termination site are used. Plasmid DNA is precipitated into 1.25 mg of 1-2 μm gold particles using 25 μl of 2.5 M $CaCl_2$ and 10 μl of 0.1 M thiamine (free base). DNA-coated particles are washed with 125 μl of 100% ethanol and then resuspended in 30 μl ethanol. The samples are sonicated to obtain an efficient dispersion, and the samples are aliquoted to obtain delivery disks containing 3 μg of DNA each. Particle bombardment is optimized according to the specific tissue to be transformed. Tissue samples are placed in Petri dishes containing 4.5 g/l basal MS salts, 1 mg/l thiamine, 10 mg/l myoinositol, 30 g/l sucrose, 2.5 mg/l amphotericin and 10 mM K_2HPO_4 at pH 5.7. After bombardment the petri dishes are incubated for 18 - 24 hours. Tissue is regenerated in plates with growth media containing the selective marker. Rooting is initiated and transformed plants are grown under optimal growth conditions in growth chambers. After 2 - 4 weeks the seedlings are transferred to new growth medium for 2 weeks and then transferred to small pots containing vermiculite. At senescence the seeds are collected from single plants. These seeds are germinated and used to assess salt tolerance of the transgenic plants.

Example 5

Overexpression of AtNHX1-homologs in other plants.

Overexpression of AtNHX1-homologs from other plant species, preferably salt tolerant species (i.e., sugar beet) in other plants (potato, tomato, brassica, cotton, sunflower, strawberries, spinach, lettuce, rice, soybean, corn, wheat, rye, barley, atriplex, salicornia, and others) is achieved by *Agrobacterium tumefaciens*-based transformation and/or particle bombardment as described above (in Examples 3 and 4). Regeneration of the transformed plants is performed as described in Examples 3 and 4 (AtNHX2, AtNHX3 or AtNHX4).

Example 6

Expression of PNHX, AtNHX1, AtNHX1 homologs and AtNHX1 derivatives in *Saccharomyces cerevisiae*.

Expression of TNHX or PNHX, preferably AtNHX1, AtNHX1 homologs (such as

AtNHX2, AtNHX3, AtNHX4), and AtNHX1 derivatives in yeast is useful to assess and characterize the biochemical properties of the recombinant and native polypeptides. Expression in yeast also facilitates the study of interactions between AtNHX1, its homologs and derivatives with regulatory polypeptides. We have made conditional expression constructs by ligating the coding region of the AtNHX1 cDNA into two vectors, pYES2 (Invitrogen) and pYEP434 [35]. Both constructs provide galactose-inducible expression, but pYES2 has a URA3 selectable marker while pYEP434 has LEU2 as a selectable marker. Transformation by lithium acetate [36], 1994), is followed by selection on solid media containing amino acids appropriate for the selection of cells containing the transformation vector. For integrative transformation, the YXplac series of vectors for integrative transformation are used [37].

Example 7

Molecular characterization and functional analysis of Na^+/H^+ exchangers from Arabidopsis and other plants, preferably salt-tolerant (halophytes) plants.

We do molecular and biochemical characterization of the different Na^+/H^+ exchangers from *Arabidopsis* and other plants, preferably salt tolerant plants (halophytes) . We determine the expression patterns of the different *Arabidopsis* putative exchangers. Using Northern blot analysis with isoform-specific cDNA probes under high stringency conditions and standard molecular biology protocols, we determine the tissue-specificity, developmental and salt-inducibility gene expression profiles of each isoform.

We employ common molecular biology procedures to isolate Na^+/H^+ exchangers from other plants (Table 5), in particular halophytes (such as *Beta vulgaris*, *Atriplex, Messembryanthemum chrystalinum,* etc.). We designed degenerate oligonucleotide PCR primers, based upon highly conserved regions within Na^+/H^+ exchangers (one within the amiloride-binding domain, and another within a region about 200 amino acid residues further downstream) from *Arabidopsis,* yeast, mammals, and *C. elegans*, to generate a 600 – 1,000 bp DNA fragments by PCR. Sequencing of these products revealed significant homology to AtNHX1 and they are therefore being used as a probe to screen the different halophyte cDNA libraries to isolate the full-length cDNAs by standard methods. We use the nucleic acid molecules obtained in this procedure in methods of producing transgenic host cells and plants as described above.

We have subcloned unique regions from AtNHX1, AtNHX2 and AtNHX3 isoforms

into a prokaryotic expression vector (pGEX2TK, Pharmacia) for the production of recombinant GST-fusion proteins that are being used for the generation of isoform-specific polyclonal antibodies in rabbits. Briefly, sequence-specific oligonucleotides, with 5' BamHI (sense strand) and 3' EcoRI (antisense strand) flanking restriction sites, were used for PCR-mediated amplification of the unique (partial) coding regions from each isoform, and the digested PCR products were ligated into EcoRI/BamHI-digested pGEX2TK vector. pGEX2TK plasmids containing the inserts corresponding to each AtNHX isoform were sequenced on both strands to verify the fidelity of the PCR reaction and were used for expression and purification of the recombinant GST-fusion proteins in *E.coli* (BL21pLysS) as per manufacturers instructions (Pharmacia). We follow an identical procedure to that described above to produce recombinant halophyte-PNHX GST-fusion protein in *E. coli.* Antibodies against the fusion proteins are produced in rabbits by standard procedures and their isoform-specificity are confirmed by western blotting using the different GST-fusion proteins. The antibodies are used in conjunction with subcellular membrane fractions (prepared from sucrose density gradients) [15] from various *Arabidopsis* and other plant tissues, preferably halophyte tissues and western blots to determine the subcellular localization of each Na^+/H^+ exchanger isoform. These localization studies assign functions to the various isoforms.

Example 8

Biochemical characterization and functional analysis of Na^+/H^+ exchangers from Arabidopsis and other plants, preferably salt-tolerant (halophytes) plants.

Biochemical characterization of the Na^+/H^+ exchanger isoforms is performed in (i) heterologous eukaryotic expression systems (baculovirus expression system in Sf9 insect cells, transgenic yeast); and in (ii) transgenic plants.

The use of heterologous expression systems allows the fast characterization of the kinetic properties of each exchanger isoform (K_m, V_{max}, ion specificity). Baculovirus-infected Sf9 cells have proven to be a useful and adaptable system for high-level expression of correctly folded eukaryotic membrane proteins, thus they are an ideal tool for the study of membrane-bound proteins. The large size of the cells, combined with the relatively short time needed for the expression of the foreign plasma membrane-bound proteins (3-4 days) provides an excellent experimental system for the application of isotope exchange techniques. For expression in Sf9 insect cells, the Invitrogen baculovirus Sf9 insect cell

system is used. Expression vector constructs (pBluBac4.5, Invitrogen) encoding full-length AtNHX exchanger proteins are prepared for each AtNHX and other PNHX isoforms using a PCR-based subcloning approach similar to that described above for the generation of GST-fusion proteins. Initially, the suitability of the insect cell expression system for uptake analysis is performed using a single AtNHX isoform. The other PNHX isoforms are studied in a similar manner. Cultures of Sf9 insect cells are infected with baculovirus containing expression vector constructs encoding the different PNHX isoforms. Infection and selection of transformants are performed as per manufacturer's instructions (Invitrogen). The isoform-specific antibodies described above aid in the assessment of recombinant protein expression and localization within the insect cells.

Equally important is the use of transgenic yeast as a tool for the expression of recombinant eukaryotic proteins, particularly because of post-translational modifications and targeting to endomembranes. In addition, functional complementation of yeast mutant strains with plant proteins is often possible. We have subcloned the AtNHX1 cDNA into a yeast expression vector (pYES2) using a PCR-based approach as described above. Yeast (strain w303a) have been transformed with this construct and expression of the recombinant plant protein is confirmed once the antiserum is available. In addition, salt-tolerance of transformed yeast isassessed for each AtNHX isoform by comparing growth rates at different NaCl concentrations. Methods for the isolation of transport-competent plasma membranes and tonoplast and the isolation of intact vacuoles are performed. The kinetics of H^+/Na^+ exchange is measured in intact insect cells and yeast, intact yeast vacuoles, and isolated plasma membranes and tonoplast vesicles according to known methods. Na^+ influx in intact cells is monitored by isotopic exchange using $[^{22}Na^+]Cl$ and fast-filtration techniques [17,i,ii]. Kinetics of H^+-dependent Na^+ fluxes in vesicles is monitored by following the pH-dependent fluorescent quench of acridine dyes [13,17].

The results of these kinetic characterization studies provides information about the ion specificity, affinity, and optimal activity conditions for each AtNHX isoform. We assign the activity of each isoform to the corresponding target membrane, andwe also determine which of the isoforms have a higher affinity for sodium. We characterize the mechanisms of salt tolerance in general and tissue-specificity and developmental expression in particular.

In transgenic plants, expression of the different Na^+/H^+ antiports is verified with western blots using the isoform-specific antibodies described above. The kinetics of H^+/Na^+ exchange is measured in intact vacuoles, isolated plasma membranes and tonoplast

vesicles (from roots and leaves) as described above.

Example 9

Identification of positive and negative regulators of Na^+/H^+ antiport activity.

Heterologous expression of plant transport molecules in *Saccharomyces cerevisiae* has been used successfully in recent years in numerous studies. The availability of yeast mutants with salt-sensitive phenotypes (generated by 'knock-outs' of sodium transport molecules such as *Δena1-4* - the plasma membrane Na^+- ATPase pumps) makes it an especially suitable system for the study of sodium transport molecules. This heterologous expression facilitates kinetic studies of the antiport activity in yeast cells using radiolabelled $^{22}Na^+$.

Successful suppression of yeast mutants, incapable of sodium detoxification allows for the genetic identification of positive and negative regulators of these Na^+/H^+ antiports. Mutant yeast cells having a suppressed phenotype as a result of the expression of a plant Na^+/H^+ antiport are transformed with an *Arabidopsis* cDNA library for the purpose of identifying particular regulators of these antiport molecules. A phenotype of increased sodium tolerance in yeast identifies particular positive regulators of the antiport activity while negative regulators are identified by a phenotype of decreased sodium tolerance. These phenotypes depend on the co-expression of the particular cDNAs identified along with that of the Na^+/H^+ antiport under investigation. Identification of essential amino acid residues regulating the activity of Na^+/H^+ exchanger molecules is investigated by random mutagenesis of the antiport molecule which is achieved by PCR using a commercially available low fidelity *Taq* enzyme. The constructs generated are used in transforming sodium-related yeast mutants to identify particular Na^+/H^+ antiport residues that affect suppression of the mutant yeast phenotype. Both gain-of-function and loss-of-function mutations are examined and mapped to the particular mutant residue by sequencing. Gain-of-function mutations are of particular interest since they represent constitutive activation of the antiport activity allowing for increased sodium detoxification.

Example 10

Transformation of Arabidopsis thaliana using overexpression of different putative isoforms and antiports from other plants, preferably salt tolerant plants and evaluation of salt-tolerance.

Arabidopsis represents a readily transformable model organism with the particular

advantage of having a short generation time. *Agrobacterium tumefaciens*-mediated genetic transformation is utilized for *Arabidopsis* (ecotype Columbia). Studies include the overexpression of PNHX transgenes in a wild-type background, combined overexpression of more than one PNHX transgene, and suppression of endogenous PNHX expression using antisense PNHX expression. Stable transformation of progeny is confirmed by Southern blotting. Overexpression of transgenes, or suppression of expression using antisense constructs, is confirmed by Northern and western blotting. In all cases, salt-tolerance of transgenic plants is compared to wild-type plants, and control plants transformed with empty transformation vectors. Separate transformations are performed on *Arabidopsis* plants using expression vector constructs for each of the different AtNHX isoforms. In addition, *Arabidopsis* plants are transformed with PNHX genes from other plants, preferably salt tolerant plants in order to assess the effect on salt tolerance of the expression of a Na^+/H^+ exchanger in a glycophytic plant.

For overexpression studies, full-length AtNHX1, AtNHX2, AtNHX3 and AtNHX4 cDNAs are subcloned in a sense orientation into the expression vector containing a "superpromoter" [20]. A PCR based subcloning strategy is used for each AtNHX cDNA as described above for the production of NHXGST-fusion constructs. For the production of vector constructs containing PNHX cDNAs in an antisense orientation, oligonucleotides with SalI and SacI restriction sites flanking the C-terminal and N-terminal PNHX regions respectively, are used for PCR amplification. All plasmid constructs are sequenced on both strands to confirm the fidelity of the PCR amplification before transformation of *Agrobacterium tumefaciens* (strain LBA4404). For each PNHX-pBISN1 construct, approximately 1L of *Agrobacterium* culture, grown under antibiotic selection at 28°C, is used for the transformation of *Arabidopsis*. Plants are ready for transformation when primary bolts are approximately 15cm. About 2 flats of plants (~ 80 plants per flat) are used per transformation. A highly efficient, vacuum-less infiltration transformation method [iii] is used. Harvested *Agrobacterium* cultures are resuspended in an infiltration media containing a mild surfactant (Silwet L-77, Lehle Seeds), and each pot of *Arabidopsis* is simply submerged in the *Agrobacterium* for 2-6 minutes. Plants are thereafter drained, and returned to the growth chamber until the seeds are ready for harvesting (about 4 weeks). Seeds (T1 generation) are collected and after surface sterilization, are plated on sterile, selective media containing kanamycin, vernalized, and then grown under optimal conditions. Healthy seedlings showing kanamycin resistence after about 7 days are

transplanted to soil and the presence of the transgene confirmed by Southern blotting. Seeds from T1 transformants (ie T2 generation) are harvested, sown, and T2 plants used for Northern and western blotting to determine the expression patterns of the transgenes and PNHX proteins. Representative transgenic lines (e.g. showing low, medium, or high transgene expression) is used for studies of salt-tolerance. A similar approach is used for transformation of *Arabidopsis* with the PNHXs from other plants.

Salt tolerance is assessed by measuring the growth rate of the plants at increasing salt concentrations. Plant biomass, root/shoot ratios, tissue ion content is measured. Root and hypocotyl growth rates is measured and correlated with tissue water content of plants growing at different NaCl concentrations.

Example 11

Transformation of crop plants with *A. thaliana* and/or other exchangers under constitutive and inducible promoters and evaluation of salt-tolerance.

a) *Agrobacterium tumefaciens*-mediated transformation of crop plants

We assess whether or not homologues of the AtNHX genes exist in the plant of choice. We use degenerate oligonucleotide PCR-primers (as described for other plants) and a cDNA library to isolate the full-length cDNA. The high efficiency *Agrobacterium*-mediated transformation method developed specifically for *Brassica* by Moloney et al [iv] is used to introduce and overexpress foreign nucleic acid molecules and/or overexpress the endogenous PNHX nucleic acid molecule in the crop plant(s). This method takes advantage of the fact that cut cotyledonary petioles from, which are capable of undergoing organogenesis (ie generating explants), are very susceptible to *Agrobacterium* infection. Shortly after germination (~ 5 days) cotlyedons are excised and imbedded into Murashige-Skoog medium (Gibco) enriched with benyzladenine. Expression vector constructs are prepared using a PCR-based subcloning approach as described above using the pCGN5059 binary plasmid (which employs the CaMV 35S promoter to drive constitutively high expression) engineered for gentamycin resistance [iv] and cDNAs of the various AtNHX clones and/or the halophyte PNHX clones, and the choosen plant PNHX clones. Excised cotyledons are infected with *Agrobacterium* cultures (strain EHA101), containing the vector construct of interest, by brief dipping and then co-cultivated with the *Agrobacterium* for a 72h. Subsequently, cotlyedons are transferred to regeneration medium containing gentamycin as the selective agent. After explant regeneration, and

subculturing, on selective media (~ 4 weeks) explants are transferred to rooting medium and then into soil once a root mass has developed. Tissue samples are examined from growing plants to confirm transgene presence by Southern blotting as described above for the transformation of *Arabidopsis*. Transformed plants (T1 generation) are allowed to flower and set seed and these seeds are germinated (T2) under selective conditions and transformants used for expression analysis of the transgenes and evaluation of salt-tolerance as described above. Also, biochemical analysis of the plants is performed. These include, Na^+/K^+ ratios, sugar, amino acid and quaternary N-compounds. Salt-tolerance is also evaluated in fields trials.

b) Microprojectile bombardment-mediated transformation of crop plants.

A microprojectile bombardment-mediated transformation of crop plants is used when *Agrobacterium tumefaciens*-mediated transformation is not successful. We assess whether or not homologues of the AtNHX genes exist in the plant of choice. We use degenerate oligonucleotide PCR-primers (as described above) and a cDNA library to isolate the full-length cDNA. Expression vector constructs, using the pBAR vector for high level expression of AtNHX or the halophyte PNHX or the endogenous PNHX from the plant of choice, are used in conjunction with the microprojectile bombardment system as described by Tomes et al. [v]. Bombardment procedures is carried out in callus tissue. Plant calli are initiated by culturing immature embryos on Callus medium [vi]. After about 2 weeks, friable calli that are growing rapidly are subcultured and grown for an additional 2 weeks and then used for transformation. Calli for transformation are transferred to fresh medium, incubated for 24 h and bombarded with tungsten microprojectiles carrying the pBARNHX vector construct. Bombardment conditions is performed according to manufacturer's instructions. Calli that show visible growth 10 days after bombardment are transferred to selective media (containing either Bialaphos or Ignite) in order to identify putative transformants. The growth of transformed plant calli on this selective media is continued for 3-4 months. Each putative stable transgenic event becomes apparent as a mass of friable embyogenic callus growing in the presence of the selection agent. Stable transformation is verified by Southern blots. Selected calli are transferred onto a regeneration medium [v], kept in the dark at 28°C for 7 days and then transferred to growth chambers under a 16-h photoperiod until green shoots appear. Plantlets (1-2 cm long) are transferred to individual tubes containing germination medium to allow continued development. At the three to four leaf stage, plants are transferred to soil and into the

greenhouse. At the eight-leaf stage, these plants are sprayed with 1% (w/v) Ignite herbicide to detect the presence of the BAR gene. This herbicide kills those plants not carrying the BAR gene. Confirmed transgenic plants (T1) are allowed to mature, flower, set seed, and seeds used for the production of T2 plants. Transgenic T2 plants are used for the evaluation of salt-tolerance as described above. Transgenic T2 and T3 plants are used in field trials for the evaluation of salt tolerance.

METHODS

Cloning of the Arabidopsis Na^+/H^+ antiport cDNA (AtNHX1)

The full-length cDNA of AtNHX1 was cloned by us from an *Arabidopisis thaliana* (Columbia) seedling cDNA library [38]. The library was initially screened with an EST (GenBank # T75860; Figure 8(h)) obtained from the Arabidopsis Biological Resource Center (ABRC) that showed homology to Arabidopsis genomic sequence (A-TM021B04.4). The invention includes nucleic acid molecules of between about: 500-1000, 1000-1500 1500-1600, 1600-1700, 1700-2000 or 2000-2500 or greater than 2500 nucleotides including the EST sequence (or a sequence having at least about: 35, 35, 55, 65, 75, 85, 90, 95, 99, 99.5 sequence identity to the EST sequence or the polypeptide encoded by the EST sequence) and which encodes a polypeptide that extrudes monovalent (preferably potassium ions or lithium ions, most preferably sodium ions) out of the cytosol for preparation of transgenic plants and host cells, and in the other methods of the invention described below. These sequences are useful in the methods of the invention described above (for example as a probe, research uses, hybridization). The Arabidopsis genomic sequence predicted a polypeptide of 457 amino acids. Plaques that hybridized with the labeled EST probe were subjected to a secondary screen using the PCR product from the nested amplification of a region coding for the N-terminal portion of the predicted polypeptide. The forward primer, based on the predicted start codon of the polypeptide (Primer-NT), 5-GCCATGTTGGATTCTCTAGTGTCG-3 and the reverse primer, based on the stop codon predicted from the EST (Primer-CT), 5'-CCGAATTCTCAAAGCTTTTCTTCCACG-3', were used to amplify a 1.7 kb product from the seedling library. This product was purified by agarose gel electrophoresis and used as the template for a second amplification using primer-NT and a reverse primer (primer-C) based on the genomic sequence, 5'-CGGAATTCACAGAAAAACACAGTGAGGAT-3'. The resulting 900 bp fragment served as the template for the probe used in the secondary screen. The pure plaques obtained in the secondary screen were tested by PCR using the

primer-NT, primer-CT combination. Three of the plaques, from which a 1.7 kb product was amplified, were selected for excision of the phagemid. Single colonies containing the excised phagemid were grown in liquid culture. Aliquots of each of these cultures were used as templates for the PCR amplification of the region bound by the library plasmid to the 5' side of the clone (T3 promoter) and the reverse primer C. In one clone, a 1.2 kb fragment was amplified, which implied that the clone had an upstream untranslated region of approximately 300 bp. This clone was selected for complete sequencing.

Cloning of the *Arabidopsis* AtNHX2 Na^+/H^+ antiport cDNA

The full-length AtNHX2 cDNA was cloned from an Arabidopsis thaliana (Columbia) seedling cDNA library. PCR primers were designed for the amplification of the AtNHX2 sequence based on a BAC DNA sequence (MTE17) with a predicted amino acid sequence showing homology to AtNHX1. The forward primer (X6F), 5'-CCTCAGGTGATACCAATCTCA-3' and the reverse primer (X6REV), 5'-GATCCAATGTAACACCGGAG-3' were used to amplify a 1.2 kb product from the seedling library by PCR. This product was purified by agarose gel electrophoresis and used as a probe in hybridization screening of the seedling cDNA library. Plaques that hybridized with the labeled probe were subjected to a secondary screen using the 1.2 kb PCR product as a probe. Pure plaques obtained in the secondary screen were tested by PCR using primer - X6F, primer - X6REV combination. Only one of the plaques had the 1.2 kb product amplified from it. This plaque was used for excision of the phagemid. This clone was used for complete sequencing.

Cloning of the *Arabidopsis* AtNHX3 and AtNHX4 Na^+/H^+ antiport cDNAs

Full length AtNHX3 and AtNHX4 cDNAs were cloned by us from an Arabidopsis thaliana (Columbia) seedling cDNA libraries (CD4-15 and CD4-16; Arabidopsis Stock Center, Columbus, Ohio). PCR primers were designed for the amplification of a genomic sequence based on a BAC DNA sequence (F20D21) with a predicted amino acid sequence showing homology to both AtNHX1 and AtNHX2. The forward primer (NHX7F), 5'-TTCGTTCTCGGCCATGTCC-3' and the reverse primer (NHX7REV), 5'-CGGAGAGACCAACACCTTCTGC-3' were used to amplify a 2.2 kb product using *Arabidopsis thaliana* (Columbia) genomic DNA as a template. This product was purified by agarose gel electrophoresis and used as a probe in hybridization screening of the seedling cDNA libraries. Plaques that hybridized with the labeled probe were subjected to a

secondary screen using the 2.2 kb PCR product as a probe. Pure plaques were used as templates for the PCR amplification of the region bound by the library plasmid using the T3 and T7 promoter sequences as primers. Two independent clones (insert sizes of 1.7 kb and 2.1kb) were selected for phagemid excision and complete sequencing.

Southern Blot Analysis

Genomic DNA was isolated from mature leaf tissue of *Arabidopsis thaliana* (Columbia). 10 ug of this genomic DNA was digested with ClaI, EcoR1, XbaI, or HindIII, fractionated on 0.7% agarose gel, and transferred to Hybond N^+ membrane (Amersham) according to manufacturers instructions. Overnight hybridization was performed at 65°C in Amersham hybridization buffer with AtNHX1 cDNA fragments labeled with ^{32}P by the random priming method. The final wash was in 0.1X SSPE, 0.1% SDS at 65°C. Hybridization signals were detected by autoradiography on BioMax hyperfilm (Kodak).

Northern Blot Analysis

Arabidopsis thaliana ecotype Columbia was grown either on vertical plates on medium containing 0.5X MS salts and 1% agar at 20-25°C under continuous fluorescent light for 1.5 weeks or in soil at 20-25°C under fluorescent light and incandescent light with a 14 hour photo period for 3-4 weeks. Total RNA was isolated from flower, leaf, and inflorescence stems of the mature plants and from root and shoot tissues of the vertically grown seedlings using TRIZOL reagent (GibcoBRL). 40 ug of RNA was electrophoresed and transferred to Hybond N^+ membrane (Amersham) according to manufacturers instructions. Methylene blue was used to visualize the 26S and 18S ribosomal RNA for quantitation. The blotted RNA was hybridized and washed as described for the southern blot analysis.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

Generation of Transgenic Arabidopsis Plants Overexpressing AtNHX1

An AtNHX1 PCR product was amplified using Vent DNA polymerase (New England

Biolabs) with the following primers (SE-ATX1-SalI 5'-CGCGTCGACATGTTGGATTCTCTAGTGTCG-3' and ATXCT2 5'-CCGAATTCTCAAAGCTTTTCTTCCACG-3'). This product was digested with SalI gel purified and used in a ligation reaction along with pBISNI prevously digested with SalI and SmaI and gel purified. The resulting vector pBISNI-AtNHX1 contained the AtNHX1 open reading frame in a sense orientation under the control of the super promoter.

Overexpression constructs carrying the *AtNHX1* cDNA are introduced into an electro-competent *Agrobacterium tumefaciens* (LBA4404) by electroporation. The *Agrobacteria* are plated on LB plates containing 50 mg/L kanamycin and grown for ~2 days at 30 ^{o}C to select for bacteria carrying the overexpression constructs. One liter liquid LB+kanamycin (50mg/L) is inoculated with a single *Agrobacterium* colony selected from the LB (kanamycin 50mg/L) plates. The culture is grown to a minimum of OD=1 (600nm) for 2-3 days. The *Agrobacteria* are then pelleted and resuspended in 1L infiltration medium (IM - 0.5XMS salts; 0.5 g/L MES; 5% sucrose; 0.03% Silwet L-77). Flowering *Arabidopsis* plants with primary bolts reaching ~15cm are used for the transformation procedure (T1). Pots of *Arabidopsis* plants are dunked into the IM solution containing the *Agrobacteria* and left submerged for 2-6 minutes. The same procedure can be repeated after 8-12 days on the same plants. Plants are allowed to senesce, the plants are dried thoroughly and the seeds harvested. Seeds are plated on agar plates containing 25 mg/L kanamycin in a medium containing MS salts, 0.8% (w/v) agar adjusted to pH 6.0 with KOH. The plates are transferred to a growth room at 25 ^{o}C under continuous light. After 10 days the kanamycin-resistant seedlings (T2) are transferred to small pots containing vermiculite. At senescence (~8 weeks) the seeds are collected from single plants and plated on agar plates containing MS salts and 25mg/L kanamycin. After 10 days the kanamycin-resistant seedlings (T3) are transferred to small pots containing vermiculite. Seeds produced by these plants are germinated and used to assess salt tolerance of the transgenic plants.

Assessment of Salt Tolerance in Transgenic Plants

This procedure is described in the legend for Figure 7.

The present invention has been described in detail and with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope of the

invention.

All articles, patents and other documents described in this application (including Genbank sequences and/or accession numbers), US application no. 60/078,474 (filed March 18, 1998), US application no. 60/116,111 (filed January 15, 1998) and US patent nos. 5,612,191, 5,763,211, 5,750,848 and 5681714, are incorporated by reference in their entirety to the same extent as if each individual publication, patent or document was specifically and individually indicated to be incorporated by reference in its entirety. They are also incorporated to the extent that they supplement, explain, provide a background for, or teach methodology, techniques and/or compositions employed herein.

High stringency (very similar sequences)	
Hybridization	**Wash**
55 – 65 °C 5xSSC 2% SDS 100 µg/ml SSDNA	60 – 65 °C 0.1xSSC 0.1% SDS

Intermediate stringency (similar sequences) (moderate stringency)	
Hybridization	**Wash**
40 – 50 °C 5xSSC 2% SDS 100 µg/ml SSDNA	50 – 50 °C 0.1xSSC 0.1% SDS

Low stringency (low similarity among sequences, i.e. many sequences similar)	
Hybridization	**Wash**
30 – 40 °C 5xSSC 2% SDS 100 µg/ml SSDNA	40 – 50 °C 2xSSC 0.2% SDS

Abbreviations:

SSC = sodium chloride-sodium citrate buffer
SSDNA = single stranded DNA

Table 4

Table 5 - List of Plants

Alfalfa
Almond
Apple
Apricot
Arabidopsis
Artichoke
Atriplex
Avocado
Barley
Beet
Birch
Brassica
Cabbage
Cacao
Cantaloup/cantalope
Carnations
Castorbean
Caulifower
Celery
Clover
Coffee
Corn
Cotton
Cucumber
Garlic
Grape
Grapefruit
Hemp
Hops
Lettuce
Maple
Melon
Mustard
Oak
Oat
Olive
Onion
Orange
Pea
Peach
Pear
Pepper
Pine
Plum
Poplar
Potato
Prune
Radish
Rape
Rice
Roses
Rye
Sorghum
Soybean
Spinach
Squash
Strawberries
Sunflower
Sweet corn
Tobacco
Tomato
Wheat

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